Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Population diversity and transmission dynamics of *Plasmodium* sp.

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor no Ramo de Ciências Biomédicas, Especialidade em Parasitologia, realizada sob orientação científica da Invª. Doutora Ana Paula Arez

Cristina Isabel Rodrigues Mendes

ABRIL, 2014

Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Population diversity and transmission dynamics of *Plasmodium* sp.

Cristina Isabel Rodrigues Mendes

Licenciada em Química Aplicada pela Universidade Nova de Lisboa

Mestre em Biotecnologia pela Universidade Nova de Lisboa

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor no Ramo de Ciências Biomédicas, Especialidade em Parasitologia, realizada sob orientação científica da Inv[a]. Doutora Ana Paula Arez

Orientador: Inv[a]. Doutora Ana Paula Arez
Unidade de Parasitologia Médica
Instituto de Higiene e Medicina Tropical

Co-Orientador: Inv. Doutor Pedro Berzosa
Centro Nacional de Medicina Tropical
Instituto de Salud Carlos III

Comissão Tutorial: Prof. Doutor Bruno de Sousa
Faculdade de Psicologia e de Ciências da Educação
Universidade de Coimbra

O trabalho foi financiado pela Fundação para a Ciência e Tecnologia, através da bolsa de doutoramento SRFH/BD/41473/2007, pelo PEst-OE/SAU/LA0018/2011 - Proj. Estratégico LA0018 2011/2012 e pelo projeto de investigação PTDC/SAU-EPI/113326/2009.

ABRIL, 2014

Scientific articles

Results presented in this thesis have been published or are in preparation for publication:

Published:

Mendes C., Dias F., Figueiredo J., Mora V.G., Cano J., de Sousa B., do Rosário V.E., Benito A., Berzosa P., Arez A.P. (2011) Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLos Neglected Tropical Disease;* 5(6):e1192.

Mendes C., Salgueiro P., Gonzalez V., Berzosa P., Benito A., do Rosário V.E., de Sousa B., Cano J., Arez A.P. (2013) Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea. *Malaria Journal*; 12:114

In preparation:

Mendes C., Custódio A., Pinto J., Arez AP., Silveira H. Molecular evidence of positive selection in transglutaminases of *Anopheles gambiae.*

Aos meus pequenotes,

Mada e Dudu ☺…

Agradecimentos

O presente trabalho não teria sido possível sem a colaboração de várias instituições e pessoas, às quais eu gostaria de agradecer:

Ao Instituto de Higiene e Medicina Tropical e ao Centro de Malária e outras Doenças Tropicais, de Lisboa, que me deram todas as condições necessárias para que eu conseguisse desenvolver o meu trabalho. Queria agradecer à "antiga" Unidade de Malária, onde desenvolvi o meu trabalho e especialmente ao Professor Virgílio E. do Rosário por todo o apoio dado como diretor desta unidade, durante os anos de doutoramento.

Al Centro Nacional de Medicina Tropical del Instituto Carlos III, de Madrid, por acogerme como estudiante y haberme permitido desarrollar la parte inicial de mi trabajo experimental, la cual incluyó la extracción de muestras biológicas, la detección e identificación de *Plasmodium* y el estudio de las mutaciones asociadas a la resistencia a distintos antimálaricos.

À Fundação para a Ciência e Tecnologia (FCT) pela concessão da bolsa de doutoramento (SRFH/BD/41473/2007), ao PEst-OE/SAU/LA0018/2011 - Proj. Estratégico LA0018 2011/2012, aos fundos concedidos no âmbito do projeto PTDC/SAU-EPI/113326/2009 e finalmente ao Instituto de Salud Carlos III, Ministério da Ciência e Inovação, Madrid, que tornaram possível a realização deste trabalho.

A todas as pessoas da Guiné-Equatorial e Angola que aceitaram participar neste estudo.

À Invª Doutora Ana Paula Arez, por ter aceite ser minha orientadora. Agradeço toda a orientação, ideias e discussões que foram trocadas ao longo destes anos, que me deram a oportunidade de crescer como investigadora. Agradeço também toda a confiança depositada em mim.

Al Doctor Pedro Berzosa Diaz, mi codirector, por aceptarme como estudiante y ofrecerme su apoyo, confianza y orientación para el buen desarrollo de esta tesis.

Al Doctor Agustin Benito Llanes, director del Centro Nacional de Medicina Tropical, Instituto de Salud Carlos III, por darme la oportunidad de trabajar en su centro y favorecer mi formación como investigadora.

Ao Prof. Doutor Bruno Sousa, por ter aceite fazer parte da minha comissão tutorial. Por ter estado sempre disponível para discutirmos todos os pormenores do tratamento estatístico.

Ao Prof. Doutor Henrique Silveira, por apesar de não ser meu orientador, ter estado sempre presente e por ter demonstrado sempre uma grande disponibilidade para me receber, trocar ideias e discutir resultados. Um muito obrigado pelo apoio e incentivo.

A mis colegas de trabajo en ISCIII-Madrid, especialmente a Vicen, por su preciosa ayuda en el laboratorio; a Paco y Carlos por ofrecerme su amistad y apoyo en todo momento - !Muchísimas Gracias!; a Patricia, Carmen, Aida, Maria, Pilar, Laura por todo el apoyo y por largas conversaciones en la comida ☺

A Jorge Cano y a todo el personal del proyecto de Guinea por realizar la colección de muestras y porque sin ellos este trabajo no se hubiera podido llevar a cabo.

A todos os meus colegas do IHMT, especialmente à Rute Félix e à Patrícia Machado por terem estado sempre presentes, por termos partilhado alegrias, tristezas, bons resultados e algumas frustrações. Pat obrigada pela leitura da tese. Vão fazer sempre parte da minha vida. Adoro-vos!!

À Ana Catarina Alves, Dinora Lopes, outras duas grandes amigas! Obrigada pela amizade e boa disposição. Mesmo nas alturas mais difíceis foi sempre importante saber que estavam lá. Mostraram que também é possível fazer amizades no trabalho. Obrigada!!

À Cláudia Gomes, Joana Figueiredo e Mónica Guerra, as minhas "meninas" e companheiras de grupo *Mixinfect*. Obrigada pela vossa boa disposição e pela ajuda no laboratório. Foi um prazer ter trabalhado convosco!

À Patricia Salgueiro, Ana Afonso, Ana Custódio, Lara Borges e à Martinha (mini-chefe), por fazerem que os dias passados no laboratório fossem muito mais

alegres. Obrigada pela amizade, mas também por todas as discussões científicas; ajudaram-me a crescer como cientista.

À Celeste Figueiredo, por ser das pessoas mais eficientes que conheço. Por me ter ajudado sempre a tratar de toda a burocracia e também por ser uma boa amiga. Obrigada pelas conversas, punham-me sempre animada.

Aos meus amigos de sempre pela amizade. Por estarem sempre presentes mesmo quando não estamos perto… Obrigada Cata e António pelas preciosas orientações no castelhano ☺.

Aos meus pais e irmã que são o meu pilar e que ao longo de todos estes anos têm estado sempre lá para mim. Agradeço o apoio, a coragem, a força e amor incondicional… Não poderia ter uma família melhor! Adoro-vos do fundo meu coração!

Ao Zé, por ter uma paciência infinita para mim. Por ter estado sempre ao meu lado, por me apoiar incondicionalmente e por nunca ter deixado de acreditar em mim. Obrigada por seres um marido espectacular e um pai simplesmente fantástico. Sem ti, nunca teria chegado até aqui. Amo-te muito!

À Madalena e ao Dudu que são o meu mundo!

Resumo

Diversidade populacional e dinâmica de transmissão de *Plasmodium* sp.

Cristina Mendes

PALAVRAS-CHAVE: Malária, *Anopheles gambiae*, *Plasmodium sp.*, *Plasmodium vivax*, antigénio Duffy, resistência a antimaláricos, hospedeiro humano.

Apesar dos esforços desenvolvidos nas últimas décadas, a malária continua a ser um dos maiores problemas de saúde pública no mundo, sendo a principal causa de morbilidade e mortalidade principalmente na África Subsaariana.

Fazer uma análise global, que integre todos os intervenientes deste sistema complexo, que engloba três entidades biológicas, fatores socioeconómicos e ambientais, não é fácil, mas pensamos ser um ponto fulcral para um maior conhecimento sobre esta doença. Neste estudo, utilizando um conjunto completo de amostras – sangue periférico e mosquitos – pretendeu-se analisar este complexo sistema de forma abrangente.

Deste modo, este trabalho teve como principais objetivos: 1) Caraterizar as populações parasitárias circulantes nos dois hospedeiros – humano e mosquito vetor - através da identificação das espécies de *Plasmodium* presentes*;* marcadores moleculares de diversidade (*Pfmsp2*) e marcadores moleculares associados a resistência a fármacos (mutações pontuais nos genes *Pfdhps*, *Pfdhfr*, *Pfcrt* e *Pfmdr1*)*;* 2) Analisar as pressões seletivas atuantes sobre os genes associados a resistência a fármacos e 3) Analisar a diversidade de genes do mosquito vetor - *AgTG1* e *AgTG2* - tentando relacioná-los com a presença/ausência de infeção.

As amostras biológicas utilizadas para este trabalho foram recolhidas em três países diferentes: na Guiné Equatorial continental foram colhidas amostras de sangue e mosquitos adultos em duas localidades, Miyobo e Ngonamanga. Em Angola obtiveram-se as amostras de sangue em quatro localidades diferentes (Gabela, Porto Amboim, Kissala – Sumbe e Praia – Sumbe) e foram ainda usados neste estudo mosquitos adultos provenientes de Antula, Guiné-Bissau.

Em relação ao primeiro e segundo objetivos deste trabalho, foi possível constatar a presença das quatro espécies de *Plasmodium* em ambos os hospedeiros, com prevalências superiores às reportadas oficialmente, incluindo *P. vivax*, espécie que ainda não tinha sido detetada na Guiné Equatorial continental. Detetou-se igualmente indivíduos Duffy negativos infetados com duas estirpes diferentes de *Plasmodium vivax* (*P. vivax* clássico e o *P. vivax* VK247). Relativamente às mutações pontuais associadas à resistência aos antimaláricos, constatou-se que de um modo geral estas ocorriam em elevada prevalência. Verificou-se igualmente que a resistência à pirimetamina encontra-se bem estabelecida neste país, enquanto a resistência à sulfadoxina terá tido uma

introdução mais recente. Relativamente ao terceiro e último objetivo deste trabalho, constatou-se que os dois genes estudados - *AgTG1* e *AgTG2*- apresentam fortes sinais de seleção positiva, podendo estar envolvidos no reconhecimento de organismos patogénicos, e por conseguinte envolvidos numa resposta contra a infeção.

Por fim, este trabalho permitiu concluir que na Guiné Equatorial continental existem as quatro espécies de *Plasmodium*, incluindo a espécie *P. vivax* que até à data não estava descrita no país. Foi encontrada uma elevada prevalência de mutações associadas à resistência à sulfadoxina-pirimetamina, pelo que se recomenda uma contínua monitorização destas mutações. Por fim constatou-se que os genes *AgTG1* e *AgTG2* apresentam fortes sinais de seleção positiva, podendo estar envolvidos na resposta à infeção por *Plasmodium.*

Abstract

Population diversity and transmission dynamics of *Plasmodium* sp.

Cristina Mendes

KEYWORDS: Malaria, *Anopheles gambiae*, *Plasmodium sp.*, *Plasmodium vivax,* Duffy antigen, antimalarial-resistance associated genes, human host

Despite all efforts made over the past decades, malaria remains a major public health problem in the world, affecting mainly the Sub-Saharan Africa.

A comprehensive analysis that integrates all factors in this complex system, which consists of three biological entities, socio-economic and environmental factors, is not easy, but it is crucial for a better understanding of this disease. In this study, using a complete set of peripheral blood samples and mosquitoes, we intended to analyse this complex system.

So, the main objectives of this study were to: 1) Characterise the circulating parasite populations in the two hosts – human and mosquito vector - through the identification of *Plasmodium* species; molecular marker diversity (*Pfmsp2*) and drug resistance-associated markers (mutations in the genes *Pfdhps* , *Pfdhfr* , *Pfcrt* and *Pfmdr1*; 2) analyse the selective pressures acting on genes associated with drug resistance and 3) analyse the diversity of genes in the mosquito vector - *AgTG1* and *AgTG2* - trying to relate them to the presence / absence of infection.

The biological samples used in this study were collected in three different countries: blood samples were collected in mainland Equatorial Guinea (in two villages: Miyobo and Ngonamanga) and in Angola (in four different villages: Gabela, Porto Amboim, Kissala - Sumbe and Praia - Sumbe); mosquitoes were collected, also, in the two villages of Equatorial Guinea and in Antula, Guinea Bissau.

Regarding the first and second objectives of this study, it was possible to detect the presence of the four *Plasmodium* species in both hosts, with prevalence higher than officially reported, including *Plasmodium vivax*, a species that had not been previously described in this country. Duffy negative individuals infected with two different strains of *P. vivax* (VK247 and classic strains) were also found.

Concerning the molecular markers associated to drug resistance, high prevalence was found. Results also demonstrated that pyrimethamine resistance has been established for a while in mainland Equatorial Guinea as shown by several selection signatures in the parasite genome, while sulphadoxine had a more recent introduction in this country.

Finally and regarding the third and final objective of this study, it was found that the both genes studied - *AgTG1 AgTG2* - showed strong signs of positive selection.

This study revealed that the four *Plasmodium* species are present in mainland Equatorial Guinea, including *P. vivax,* specie that had not been described yet in this country. High prevalence of mutation in genes associated with resistance to the sulphadoxine-pyrimethamine combination were found, so it is recommended a close and continuous monitoring of these mutations frequency, since there is the danger of an eventual reduction in the efficacy of combined therapy. Finally it was found that the *AgTG1* and *AgTG2* genes show strong positive selection signals, which may be involved in recognition and immune response triggered by the mosquito against the invading pathogens, like *Plasmodium*.

Abbreviations

A.	*Anopheles*
ACT	Artemisinin-based Combination Therapies
AgTG1	Transglutaminase 1 of *Anopheles gambiae*
AgTG2	Transglutaminase 2 of *Anopheles gambiae*
AMPs	Antimicrobial peptides
AS	Artesunate
AS/AQ	Artesunate/Amodiaquine
BIMCP	Bioko Island Malaria Control Project
Ca^{2+}	Calcium ion
CEC1	Cecropin 1
CLIPs	Clip-domain serine proteases
CQ	Chloroquine
CSP	Circumsporozoite Protein
CTLs	C-type lectins
DARC	Duffy antigen / receptor for chemokines
DDT	Dichloro Dipehnyl Trichlroethane
DEF1	Defensin 1
G6PD	Glucose-6-Phophate Dehydrogenase
GAM	Gambicin
Glurp	Glutamate-rich Protein
GNBPs	Gram-Negative Bacteria-binding Proteins
GTP	Guanosine TriPhosphate
HbS	Haemoglobin S
He	Heterozygosity
Imd	Immune Deficiency pathway
IPT	Intermittent preventive treatment
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets
LLINs	Long-Lasting Insecticidal Nets
LRIMs	Leucine-Rich Immune Proteins
MAGs	Male Accessory Glands
MOI	Multiplicity of Infections
MSP-1	Merozoite Surface Protein 1
MSP-2	Merozoite Surface Protein 2
MSP-3α	Merozoite Surface Protein 3 alpha
NF-kB	Nuclear Factor kB
P.	*Plasmodium*
PAMPs	Pathogen-Associated Molecular patterns
PCR	Polymerase Chain Reaction

Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
Pfdhps	*Plasmodium falciparum* dihydropteroate synthase
Pfmdr1	*Plasmodium falciparum* multidrug resistance 1 gene
PGRPs	Peptidoglycan Recognition Proteins
PK	Pyruvate Kinase
PO	Phenoloxidase
PPO	Prophenoloxidase
PRR	Pattern Recognition Receptor
PYR	Pyrimethamine
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
RT-PCR	Real-Time PCR
s.l.	*sensu lato*
s.s.	*sensu stricto*
SCRs	Scavenger Receptors
SFX	Sulphadoxine
SP	Sulphadoxine-Pyrimethamine
SRPNs	Serpins
STAT	Signal Transducers and Activators of Transcription
STR	Short Tandem Repeat
TEPs	Thioester-containing Proteins
TG	Transglutaminase
WHO	World Health Organization

Table of Contents

List of Figures

Chapter 1 – Introduction

Chapter 2 - Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea

Chapter 3 - Duffy Negative Antigen Is No Longer a Barrier to *Plasmodium vivax* – Molecular Evidences from the African West Coast (Angola and Equatorial Guinea)

Chapter 4 – Molecular evidence of positive selection in transglutaminases of Anopheles gambiae

List of Tables

Chapter 1 – Introduction

Chapter 2 - Genetic diversity and signatures of selection of drug resistance in Plasmodium populations from both human and mosquito hosts in continental Equatorial Guinea

Chapter 3 - Duffy Negative Antigen Is No Longer a Barrier to *Plasmodium vivax* – Molecular Evidences from the African West Coast (Angola and Equatorial Guinea)

Chapter 4 – Molecular evidence of positive selection in transglutaminases of Anopheles gambiae

Chapter 1 –

General Introduction

I. Malaria

The understanding of malaria and its complex life cycle has increased enormously in the last years, but despite decades of research and efforts to combat it, malaria continues to be one of the main public health problems in the world, affecting mainly the poorest areas of the planet. According to the latest World Health Organization (WHO) report, this mosquito-borne disease was responsible in 2012 for 207 million clinical cases, of which approximately 81% were in the African Region, resulting in approximately 627 000 deaths, affecting primarily children under five years old and pregnant women living in sub-Saharan Africa (WHO, 2013).

Malaria is caused by a protozoan parasite from the genus *Plasmodium*, and it is known that there are five species that may affect humans – *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi.* These parasites are transmitted to humans when female mosquitoes of the genus *Anopheles* feed on human blood. There are more than 30 anopheline species that transmit malaria to humans (WHO, 2013).

I.1. Geographic distribution of malaria

During the past decade, multiple organizations – as World Bank, Global Fund, Affordable Medicine Facility-malaria, The US President's Malaria Initiative, Bill & Melinda Gates Foundation and others – have concerted efforts to combat malaria all over the world (WHO, 2013; Murray et al., 2012). The measures undertaken had great impact especially in countries with high malaria transmission and it is estimated that 3.3 million lives have been saved during this period (WHO, 2013).

Despite all these efforts, malaria remained endemic in 103 countries, causing approximately 207 million clinical cases (range 135–287 million) and 627 000 deaths (range 473 000–627 000) in 2012. Figure 1 shows that the most affected areas are the tropical and subtropical regions of the world, specially the sub-Saharan Africa, Central and South America and the Southeast Asia.

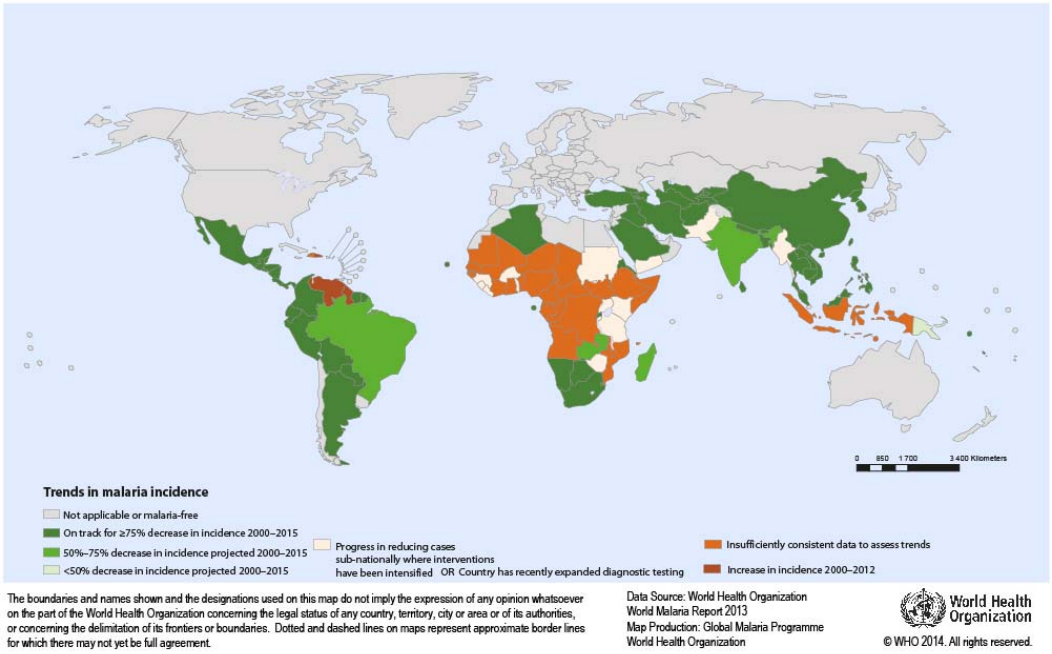

Figure 1. Trends in malaria incidence, 2000 - 2012 (from http://www.who.int/gho/malaria/en/, accessed in March 21st, 2014).

The World Health Assembly and Roll Back Malaria have as main objective to achieve a 75% reduction in malaria cases by 2015, when compared to levels in 2000. This objective, as well as all the progresses made until now, can be compromised since the international funding for malaria control has levelled off due to a reduction in the funding sources (WHO, 2013).

I.1.1. Equatorial Guinea

Equatorial Guinea is located in West Central Africa and has an area of 28 051 Km^2 and according to the most recent reports, the country has a population of about 704 000 inhabitants (https://www.cia.gov/library/publications/the-world-factbook/geos/ek.html, accessed in October 16th, 2013). This country is divided in three main regions, one continental – Rio Muni - and two islands – Bioko and Annobon. The continental area is bordered by Cameroon and Gabon; the island of Bioko is located 32 Km of Cameroon coast and is where the capital city (Malabo) is situated. The Annobon Island is located at the south of Bioko.

Equatorial Guinea has a tropical climate with distinct wet and dry seasons. The continental area presents two dry seasons - from December to March and July to September - and two rainy seasons - one stronger from September to November and other from March until late June.

In Equatorial Guinea, malaria remains the major endemic disease and the leading cause of child mortality and morbidity, being characterized as hyper- and holoendemic (Rehman et al., 2013). It is important to distinguish the insular from the continental region regarding epidemiological characteristics of malaria, since control measures have been different in different regions of the country. In 2004, the Bioko Island Malaria Control Project (BIMCP) was launched, consisting mainly in the indoor residual spraying (IRS) programme, and aiming to eliminate malaria infection in the island. The prevalence of infection has been significantly reduced (from 42% pre-intervention, to 18% in 2008) on the insular region (Pardo et al., 2006; Kleinschmidt et al., 2009) whilst the prevalence of infection remained above 50% in children under five years old in mainland region (Kleinschmidt et al., 2009).

Four species of *Plasmodium* – *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* – were present in the insular region, however in the mainland, *P. vivax* was described for the first time only quite recently (Mendes et al., 2011). In both regions, *P. falciparum* was the most prevalent species, being responsible for approximately 90% of the cases.

Concerning the mosquito vector, multiple Anopheline species were found in the continental region, *Anopheles melas* and *A. gambiae* s.s. being considered the main vectors (Moreno et al., 2004). In addition to these species, it is still possible to find *Anopheles moucheti moucheti*, *Anopheles carnevalei* and *Anopheles funestus* (Molina et al., 1993; Cano et al., 2006).

I.1.2. Guinea - Bissau

Guinea-Bissau is a country located in Western Africa, bordered by the North Atlantic Ocean, between Guinea and Senegal. With a total area of 36 125 Km^2 and a population of 1.628 603 people (https://www.cia.gov/library/publications/the-world-factbook/geos/pu.html, accessed in October 16th, 2013), the climate is characterized by

two distinct seasons: a rainy season (from June to November) and a dry season (from December to May).

In Guinea-Bissau, malaria remains a serious health problem, presenting approximately 55 000 clinical cases for year (WHO, 2013), being considered as mesoendemic-to-holoendemic with intense and seasonal transmission during the rainy season. *Plasmodium falciparum* is the most prevalent species, responsible for almost 100% of the cases although there are reports of the presence of *P. malariae* and *P. ovale* in this country (Arez et al., 2003). *Anopheles gambiae* s.s. and *A. melas* are the main malaria vectors in the area.

I.1.3. Angola

Angola, a country with 18.056 072 inhabitants and a total area of 1.246 700 Km^2 (https://www.cia.gov/library/publications/the-world-factbook/geos/ao.html, accessed in October 16th, 2013), faces several problems resulting from 27 years of civil war. Angola has shown in recent years a high growth rate due to its oil production, however much of the country's infrastructure is still damaged or undeveloped, since it is estimated that 80% of hospitals and health centres have been damaged / destroyed during the war, restricting the access to health systems to less than 30% of the population. These problems associated with many other factors as the lack of basic sanitation and difficulties in access to health centres and hospitals, allowed the spread of many diseases, including malaria.

According to the latest report, the prevalence of malaria has dropped 50% over the last five years as a result of control efforts. Nevertheless this disease remains one of the major's public health problems in Angola, being responsible for 91 deaths per 1 000 live births (President's Malaria Initiative, 2013).

Malaria is endemic all over the country, being classified as hyperendemic in the north and along the Atlantic coast. In the central and southern areas is classified as mesoendemic unstable. There are two transmission peaks, one occurring between March and May and the other between October and November. The most prevalent species of *Plasmodium* is *P. falciparum,* but the other three - *P. vivax*, *P. malariae* and *P. ovale* - are also present in this country.

There are numerous species of mosquitoes responsible for transmitting malaria parasites, *A. gambiae* and *A. funestus* being considered primary vectors (WHO, 2013).

I.2. Malaria control: antimalarials and insecticides

Despite the high number of clinical cases and deaths occurring all over the world due to malaria, this is a preventable and treatable disease. According to the WHO (2013) is essential to act at the level of transmission of the parasite by the mosquito vector, but also in the development of illness and severe disease.

I.2.1. Malaria vector control

Historically, vector control has been an important tool to reduce and even eradicate malaria in some parts of the world. Nowadays there are different options available to vector control that include chemical, biological, natural plant products, and environmental management (Raghavendra et al., 2011).

For WHO, the interventions with higher impact are insecticide-treated nets (ITNs), that include the long-lasting insecticidal nets (LLINs) and the conventional nets that are later treated with an insecticide; and IRS.

The ITNs forms a physical barrier between the infected mosquitoes and man and to be effective need to have high coverage rates. Today it is estimated that a total of 88 countries, including 39 in Africa, distribute ITNs free of charge. In fact, the percentage of households owning at least one ITN in sub-Saharan Africa is estimated to have risen from 3% in 2000 to 56% in 2012, but declined slightly to 54% in 2013, and the last data indicates that approximately 86% of the population with access to an ITN actually uses it, suggesting that efforts to encourage ITN use have been successful (WHO, 2013).

IRS with insecticides continues to be one of the main pillars for malaria control. IRS consists in the application of sprayable insecticides in the walls and roofs of the houses to kill mosquitoes. This affects the malaria transmission by reducing the life span of female mosquitoes, reducing therefore mosquito density (Raghavendra et al., 2011). In 2012, 88 countries, including 40 in the African Region, recommended IRS for

malaria control. In the last years, the proportion of at-risk population that was protected arise from less than 5% in 2005 to 11% in 2010 but fell to 8% in 2012, with 58 million people benefiting from the intervention, only in the African Region (WHO, 2013).

Another important measure is the larval control of malaria vector *Anopheles* mosquitoes. The principle of chemical larviciding is to eliminate or reduce the vector population by killing the larvae. This preventive method has been neglected, in spite of some authors thinking that should be taken into account in the new malaria control programs (Walker & Lynch, 2007). The WHO recommends larviciding only in settings where mosquito breeding sites are few, fixed, findable and easy to identify, map and treat (WHO, 2013).

I.2.1.1. Insecticide resistance

Until the early 19th century, the application of insecticides was the primary control tool in the vector control programs (Breman, 2001). Several insecticides have been used so far, but in the 20th century, after the discovery of the insecticidal potential of dichlorodipehnyltrichlroethane (DDT), a new era of insect control began. DDT was the first synthetic organic insecticide used for effective vector control; it was cheap and very efficient. However, with the extensive use of this and other insecticides, the insecticide resistances start to appear and spread in many *Anopheles* species (Raghavendra et al., 2011).

Today the insecticide resistance is one of the major problems for vector control programs and according the last report, mosquito resistance to at least one insecticide used for malaria control has already been identified in 64 countries. The one that rise bigger concern is the resistance to pyrethroids, especially in Africa.

To try to overcome this problem, WHO made a series of recommendations such as: 1) Resistance management measures should be part of every vector control program and deployed preventively, without waiting for signs of the presence of resistance or of control failure; 2) A substantial intensification of resistance monitoring is needed; 3) Using the same insecticide for multiple successive IRS cycles is not recommended; 4) In areas with high LLIN coverage, pyrethroids should not be used for IRS (WHO, 2013).

I.2.2. Malaria control

To control malaria one should take into account several aspects: first it is needed appropriate preventive measures; a good and reliable diagnostic and finally an effective treatment.

I.2.2.1. Intermittent preventive treatment

Intermittent preventive treatment (IPT) is recommended for pregnant women and for children less than five years old living in malaria endemic countries. It is estimated that a total of 36 of 45 sub-Saharan African countries had adopted IPT as national policy by the end of 2011, using sulfadoxine-pyrimethamine (SP) as the drug of reference (WHO, 2013). Several studies reported the importance of the use IPTs and ITNs during pregnancy, since leads to a reduction in stillbirths, improvements in birth weight of babies (since malaria infection during the pregnancy it is one of the main causes of low birth weight) and a reduction in the prevalence of parasitaemia and anaemia in pregnant women (Gamble et al., 2009; Eisele et al., 2012; Singh et al., 2013).

I.2.2.2. Diagnosis of malaria

Current recommendations of effective, yet expensive artemisinin-based combination therapies (ACT) for malaria in Sub-Saharan Africa have increased the importance of laboratory-confirmed diagnosis.

In the majority of malaria endemic countries many fever cases are treated presumptively with antimalarials without parasitological diagnosis; further, not all confirmed malaria cases receive appropriate treatment. It is therefore important the implementation of a universal diagnostic test, which allows to obtain reliable results.

The current reference method for malaria diagnosis is direct optical microscopic visualization of parasites on thick and/or thin blood smears (Kyabayinze et al., 2008), but unfortunately, this technique is influenced by many aspects as: the experience of the laboratory technicians, the quality of the microscopes and the lack of quality control systems (Belizario et al., 2005). To overcome these limitations, Rapid Diagnostic Tests

(RDTs) for malaria have substantial potential to help solve these questions, especially in poor areas (Reyburn et al., 2007), but it must be ensured that RDTs are highly sensitive and specific for *Plasmodium* species detection (Wongsrichanalai et al., 2007). In fact, the number of patients tested by microscopic examination increased to a peak of 188 million in 2012, whereas the number of RDTs supplied by manufacturers increased from 88 million in 2010 to 205 million in 2012 (WHO, 2013).

I.2.2.3. Malaria treatment

The first drug used to fight the high fevers caused by malaria was quinine, a medicinal plant isolated from *Chinchona* tree. In the 20th century, started to appear the first organic compounds that intend to substitute the quinine, among them were the pamaquine, quinacrine and ultimately chloroquine (CQ) (Thompson et al., 1972).

Chloroquine quickly became the drug of choice to combat malaria, since it was a cheap and effective drug. In 1955, WHO launched a campaign for malaria eradication with the wide distribution of CQ together with DDT, which produced some regional successes (Wellems & Plowe, 2001). During the late 1950s, the first cases of resistant *P. falciparum* were detected in Colombia and at the Cambodia-Thailand border (Payne et al., 1987).

Later, other antimalarial drugs were developed, such as SP that already presents some cases of resistance, particularly in the south-western Asia and South America, but still is widely used in Africa mainly for IPT; mefloquine; amodiaquine and more recently the ACTs.

Today and according to the WHO, uncomplicated *P. falciparum* infection should be treated with an ACT and currently there are 5 recommended: artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus SP and dihydroartemisinin plus piperaquine. The choice of the ACT should be based on the therapeutic efficacy of the combination in the country or area of intended use. For *P. vivax* infections, the guidelines are that it should be treated with chloroquine in areas where this drug is effective or with an appropriate ACT (not artesunate plus SP) in areas where *P. vivax* resistance to chloroquine has been documented and should include an effective schizontocidal to prevent relapse (WHO, 2013).

I.2.2.4. Antimalarial resistance

Antimalarial drug resistance is a major public health problem which delays the malaria control. Today, parasites have already developed resistance to all the drugs available for malaria control, somewhere in the world. According to the WHO, antimalarial resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bloland, 2001).

Chloroquine

The first cases of *P. falciparum* resistance were detected in Colombia and at the Cambodia-Thailand border during the late 1950s (Payne et al., 1987) and spread gradually through South America, Southeast Asia, and India in the 1960s and 1970s. In Africa the first resistance reports only appeared in the late 1970s, in Kenya and Tanzania (reviewed in Wellems & Plowe, 2001).

It is thought that CQ efficacy lies in its ability to interrupt haematin, which is released in large amounts as the parasite consumes and digests haemoglobin in its digestive food vacuole a process of detoxification as malaria parasites grow within their host's red blood cells (RBCs) (Dorn et al., 1998).

Plasmodium falciparum chloroquine resistance transporter (*Pfcrt*) is a predicted transporter that is localized in the digestive vacuole membrane and may be involved in drug efflux and/or pH regulation. Several point mutations in this gene seemed to be associated with the CQ resistance, being the N75E, K76T the most frequent in Africa and considered as the best markers (Wellems & Plowe, 2001; Le Bras et al., 2003; Bray et al., 2005).

Polymorphisms N86Y and D1246Y in the *P. falciparum multidrug resistance 1*(*Pfmdr1*) gene, that encodes for the Pgh-1 P-glycoprotein, seems to be also associated to CQ resistance (Basco et al., 1995b; Reed et al., 2000).

Sulfadoxine-Pyrimethamine

After the appearance of the CQ resistance, the antifolate combination of SP has increasingly become the drug of choice for the treatment of uncomplicated *P. falciparum* malaria. However, SP resistance has developed quickly and the first report of resistance of *P. falciparum* to pyrimethamine was in the north-east Tanzania in 1954 (Clyde, 1954). Despite the resistance reports this drug still have some efficacy in some countries of Africa and is used in the IPT.

The major cause of resistance to antifolate drugs are point mutations in *P. falciparum dihydrofolate reductase* (*Pfdhfr*) and *P. falciparum dihydropteroate synthase* (*Pfdhps*) genes that rapidly diminished their clinical effectiveness. The *Pfdhfr* is a key enzyme in the redox cycle for production of tetrahydrofolate, and the *Pfdhps* is an enzyme involved in the biosynthesis of folate (Cowman et al., 1988; Brooks et al., 1994).

Several studies showed that there are four main point mutations in the *Pfdhfr* gene that are associated to resistance to pyrimethamine (N51I, C59R, S108N and I164L) (Basco et al., 1995a; Curtis et al., 1996). The S108N mutant exhibits a low level of resistance, the N51I/S108N or the C59R/S108N double mutants, intermediate levels of resistance, and the N51I/C59R/S108N triple mutant has a higher level of resistance to this drug. Similarly, resistance to sulfadoxine is due to four mutations in the *Pfdhps* (S436F, A437G, K540E, A581G) (Brooks et al., 1994). Each successive mutation causes a decrease in the susceptibility to these drugs. In fact, the association between the mutations in the two genes, originating the quintuple mutant (*Pfdhfr:* N51I, C59R, S108N and *Pfdhps*: A437G, K540E) is associated with the clinical failure of the SP (Kublin et al., 2002; Talisuna et al., 2004).

II. Parasite

II.1. Taxonomic classification

The human malaria parasites are classified as belonging to the *Eukaryota* domain, kingdom *Protista* and phylum *Apicomplexa*, which is characterized by having

an apical intracellular complex (visible with electronic microscope) and by not having cilia or flagella, except for microgametes (Ayala et al., 1998). They belong to the *Hematozoa* class (characterized by organisms that parasitise erythrocytes); to the *Haemosporida* order (that present a mobile zygote – ookinete) and to the *Plasmodiidae* family (with two different types of multiplication in their life cycle: sexual and asexual phases). This parasite belongs to *Plasmodium* genus (characterised by having an asexual phase in cells other than erythrocytes - hepatocytes) (Knell, 1991; Ayala et al., 1998) and to *Plasmodium* and *Laverania* sub-genus. Finally, they are classified in the following species: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Antinori et al., 2012).

All species of human malaria parasites present a similar and complex life-cycle requiring two different hosts - the human and the female *Anopheles* mosquito - and showing three types of genomes: a) a nuclear genome with 14 linear chromosomes; b) a linear mitochondrial genome and finally c) a 35kb circular plastid genome that is housed in the apicoplast (Antinori et al., 2012).

II.2. *Plasmodium* life cycle

All species of *Plasmodium* that infect humans show a similar life cycle (Figure 2) which is characterised by a sexual phase, named sporogony, that take place in the mosquito vector; and an asexual phase, named schizogony, that occurs in the human host.

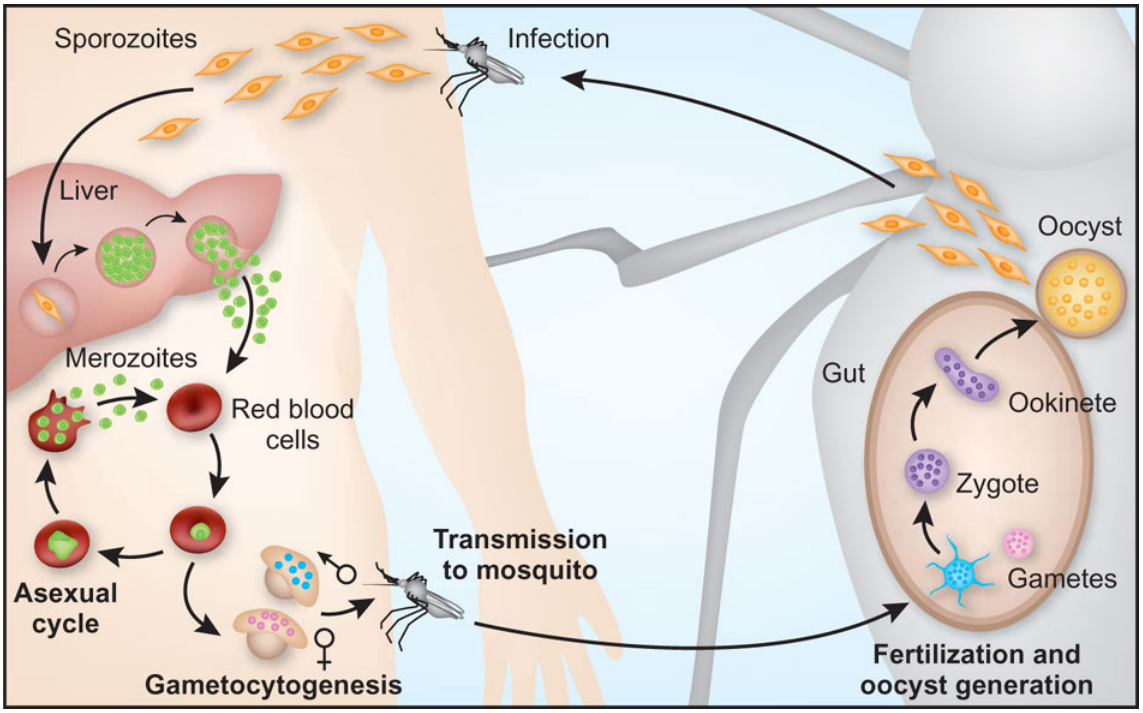

Figure 2. *Plasmodium* life cycle (adapted from Pasvol, 2010).

The *Plasmodium* life cycle starts when a female anopheline mosquito feeds on infected blood, and the gametocytes (sexual cells) began their development in the mosquito. This phase is called fertilization and corresponds to the sexual phase. The gametocytes suffer differentiation, forming the female and male gametes. Fertilization occurs between these two gametes originating a zygote (the unique diploid form of all life cycle), which undergoes meiosis and differentiation into motile ookinetes – invasive forms in the next few hours.

After approximately 24 hours, the ookinetes pass across the midgut epithelium and lodged under the basal lamina forming vegetative oocysts. These forms mature, and after several rounds of mitosis, sporozoites are formed – haploid forms. After the oocysts rupture, sporozoites are released into the hemolymph and travel through the mosquito haemacoel until reach salivary glands. When a new blood meal is taken by the mosquito female, the sporozoites are injected into the bloodstream of a new host. A few minutes later, they are already starting the invasion of the liver cells, starting the vertebrate host phase.

The newly arrived sporozoites enter the liver hepatocytes leading to liver schizonts. When they mature, merozoites are released into the peripheral blood – new invasive forms – where they will invade erythrocytes. In the case of *P. vivax* and *P. ovale*, the sporozoites can differentiate into hypnozoites, stages that can remain dormant in the liver for long periods of time.

In the erythrocytes, the merozoites begin to differentiate into trophozoites. This period is called prepatent period and its extension is characteristic of each species. After two or three days of mitotic divisions erythrocytic schizonts are formed. When erythrocytes disrupt, merozoites are released and will infect other RBCs, starting a new cycle in the blood. This phase is responsible for symptoms of malaria illness, and as the number of parasites increases (parasitaemia), the infected person becomes more severely affected (Knell, 1991; Antinori et al., 2012).

II.3. Infection dynamics

The five species of *Plasmodium* infecting humans exhibit different biological and infection characteristics. Table 1 shows some of the most important differences among them.

Table 1: Some of the most important infection characteristics of the five species of *Plasmodium* that infect humans (adapted from Antinori et al., 2012).

Characteristics	*P. falciparum*	*P. knowlesi*	*P. malariae*	*P. ovale*	*P. vivax*
Pre-erythrocytic stage (days)	5-7	8-9	14-16	9	6-8
Pre-patent period (days)	9-10	9-12	15-16	10-14	11-13
Erythrocytic cycle (hours)	48	24	72	50	48
Red blood cells affected	All	All	Mature erythrocytes	Reticulocytes	Reticulocytes
Parasitaemia per µl					
• **Average**	20 000-500 000	600-10 000	6 000	9 000	20 000-100 000
• **Maximum**	2.000 000	236 000	20 000	30 000	100 000
Febrile paroxysm (hours)	16-36 or longer	8-12	8-10	8-12	8-12
Severe malaria	Yes	Yes	No	No	Yes
Relapses from liver forms	No	No	No	Yes	Yes
Recurrences	Yes	Yes	Yes	No	Yes

One of the typical symptoms of malaria disease is the intermittent high fevers characterized by: first; a rapid rise of temperature associated with chills - the cold stage; second; a temperature peak (reaching 40-41ºC) associated with other symptoms as headache, vasodilatation and myalgia - the hot phase; and, finally, the third phase with the decreasing of temperature – the sweat stage.

The intermittent high fevers are directly related to the duration of the parasite erythrocyte cycle, and may be classified as tertian fever (caused by *P. falciparum*, *P. vivax* and *P. ovale*), with peaks of fever every three days (cycle of 48 hours), or as

quartan (caused by *P. malariae*), with peaks of fever every four days (72 hour cycle) (Knell, 1991; Carter & Mendis, 2007; Antinori et al., 2012).

For a better understanding of the *Plasmodium* species studied in the present work, a brief description of each one is present below.

II.3.1. Plasmodium falciparum

Plasmodium (Laverania) falciparum (Welch, 1896) was first observed by Charles Alphonse Laveran in 1880. Of the five *Plasmodium* species that infect humans, this is the one that causes more morbidity and mortality and present higher prevalence particularly in Africa. According to the latest WHO report (WHO, 2012) 85 countries are classified as endemic for *P. falciparum,* with 2.57 billion people at risk.

The *P. falciparum* genome sequencing in 2002, brought great advances in the knowledge of this parasite (Gardner et al., 2002). It is composed by 14 linear chromosomes, coding for 5 365 genes. However, up to now, only 1 817 have known functions.

The life cycle of *P. falciparum* is characterized by having an asexual development in the liver (pre-erythrocytic schizogony), with no hypnozoites differentiation. The first visible form in the liver appears on the fourth day after infection and corresponds to the hepatic schizont (Antinori et al., 2012).

In the erythrocytic schizogony, typically, only the young rings are visible whereas the maturation stages are rarely seen in the peripheral blood. The more developed trophozoites disappear from peripheral blood circulation as infected erythrocytes are being sequestered in the internal organs, like brain, spleen and placenta.

Another characteristic of *P. falciparum* parasite is the development of gametocytes in the internal organs, where they can be captured (Antinori et al., 2012). The gametocytogenesis of this parasite can be divided in five morphologically distinct substages (Bousema & Drakeley, 2011); Stage I – IV: Immature *P. falciparum* gametocytes are sequestered away from the circulation, Stage V: mature gametocytes are released in peripheral blood where they finally become infectious to mosquitoes

(Bousema & Drakeley, 2011; Antinori et al., 2012). The sporogonic cycle takes 9 to 10 days at a mean temperature of 28ºC.

The origin of *P. falciparum* has been object of study and until very recently, it was thought that the closest parasite of *P. falciparum* was the *Plasmodium reichenowi:* a chimpanzee parasite. The studies indicated that these two parasites have diverged at the same time 5 Myr ago (Escalante et al., 1994; Escalante et al., 1995; Rich et al., 1998; Jeffares et al., 2006); however recent works claim that *P. falciparum* is of gorilla origin (Liu et al., 2010; Holmes, 2010).

II.3.2. Plasmodium vivax

Plasmodium vivax (Grassi & Feletti, 1890) is the *Plasmodium* parasite that presents the wider distribution, being present in 109 countries considered as potentially endemic for this parasite (Guerra et al., 2010; Gething et al., 2012).

For a long period of time, *P. vivax* was considered a "benign" parasite, being neglected by the scientific community. Recently, however, this idea has changed and in the last years this parasite has become highly studied. The reasons of this change are the following: first, its wider distribution, being found in both tropical, and subtropical areas and in countries where it was not present or it was not detected by the available techniques in the past, as is the case of some countries of West and Central Africa (Poirriez et al., 1991; Snounou et al., 1998; Gautret et al., 2001; Mendes et al., 2011); second, the high number of clinical cases reported, ranging from 70 million to 300 million (Baird, 2007; Galinski & Barnwell, 2008; Mueller et al., 2009) and third, this parasite seems to be evolving and adapting, causing more severe forms of the disease including death (Genton et al., 2008; Rogerson & Carter, 2008; Tjitra et al., 2008; Alexandre et al., 2010).

In 2008, the genome of *P. vivax* was first sequenced and published (Carlton et al., 2008) and has shown that there are several differences between *P. vivax* and *P. falciparum*: the nuclear genome is higher, contains 5 433 predicted protein-encoding genes and displays chromosomes that are unique among human *Plasmodium* species with an isochore structure (Antinori et al., 2012).

Regarding *P. vivax* life cycle, sporozoites in the liver can differentiate into schizonts or into hypnozoites, which are responsible for the relapse of the infection. Hypnozoites are only formed by this species and by *P. ovale.* The sexual life cycle of this parasite in *Anopheles* mosquitoes takes 8-10 days at 28ºC (Gilles, 1993).

This parasite invades preferentially reticulocytes and all forms of the erythrocytic cycle can be found in the peripheral blood contrasting with *P. falciparum*, where only early parasites are observed (Antinori et al., 2012).

The erythrocytes invasion by this parasite has been described as being mediated exclusively by the Duffy antigen receptor for chemokines (DARC). People not presenting this antigen in the erythrocytes surface were called Duffy negative individuals [Fy(a^-b^-)] and were hypothetically resistant to *P. vivax* infection (Miller et al., 1975; Langhi et al., 2006). The small prevalence found in West and Central Africa for this parasite was attributed to the high prevalence of Duffy negative people in this region. However, recent studies demonstrate that *P. vivax* may be changing and is able to invade erythrocytes using other receptors than Duffy (Ryan et al, 2006; Cavasini et al., 2007a and 2007b; Ménard et al., 2010; Mendes et al., 2011).

II.3.3. Plasmodium malariae

Plasmodium malariae (Laveran, 1880) has a wide but sparse distribution, can be found most frequently in sub-Saharan Africa and the southwest Pacific. This parasite shows a slow development in both hosts (15 days in the *Anopheles* mosquitoes; in human, 15 days in the liver and 72 hours in the blood), and hardly causes serious forms of the disease (Collins & Jeffery, 2007).

Infections caused by this parasite rarely reach high parasitaemias (usually not exceed 30 000 parasites per microliter), probably due to a low number of merozoites produced per erythrocytic cycle. *Plasmodium malariae* does not form dormant forms in the liver, like *P. vivax* and *P. ovale*, but can persist in the blood with low parasitaemia for long periods of time (reaching 30-40years) causing recrudescence (Collins & Jeffery, 2007; Antinori et al., 2012).

The sporogonic cycle takes 14 to 16 days at 28ºC increasing the time for 30-35 days if the temperature is 20°C (Collins & Jeffery, 2007).

II.3.4. Plasmodium ovale

Plasmodium ovale (Stevens, 1922) was first discovered by Stephens in 1922 (Stephens, 1922) in an African patient. This parasite is distributed in sub-Saharan Africa, South-east Asia, Middle East, the Indian subcontinent, Papua New Guinea and East Timor Indonesia (Muller et al., 2007), and is not described as causing severe malaria cases.

This parasite can cause chronic infections, presenting low parasitaemias; and can develop hypnozoites which can cause relapses in the infection. The sporogonic cycle takes 12 to 14 days at a mean temperature of 28ºC.

Recently, two closely related but distinct species of *P. ovale* were described: *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type). It is known that despite being sympatric in both African and Asiatic regions, the existence of several geographic, temporal or ecological barriers prevent the recombination between the genomes of the two species (Sutherland et al., 2010).

II.4. Mixed Infections

Mixed infections, involving two or more species of *Plasmodium*, are very common in countries where malaria is endemic (Richie, 1988; Bruce et al., 2000). Several studies have shown that both vertebrate and invertebrate hosts may be infected with more than one species of *Plasmodium* (Mason et al., 1999; Arez et al., 2003; Mayxay et al., 2004; Snounou & White, 2004; Zimmerman et al., 2004; Marques et al., 2005; Genton et al., 2008; Bousema et al., 2008).

Concomitant infections may have effects on pathology, severity and infection dynamics, that's why it is so important a correct diagnostic.

In most of the endemic malaria countries, the diagnosis is made through blood-smears observed with a light microscopy, where parasitaemias are determined. However

this technique has some limitations, being affected by several aspects: the limit of detection is not very high (in theory, 10–100 parasites per μl) (Wongsrichanalai et al., 2007) when compared with other techniques like PCR or real-time PCR (0.05–10 parasites per μl) (Snounou et al., 2003), and is depending on the quality of the blood slides preparation, the number of microscope fields analysed and the microscopist's expertise (Zimmerman et al., 2004).

Using the standard diagnostic technique - optical microscopy – and due to the limitations referred above, mixed infections are often difficult to detect since all infections go through periods of low parasitaemia. The switch between periods of patent parasitaemia (when parasites in peripheral blood can be easily observed) and latent periods of parasitaemia (when it is not possible to observe parasites in peripheral blood), makes it difficult to distinguish the parasites only by their morphological characteristics (Richie, 1988; Snounou et al., 2004; Zimmerman et al., 2004; Rajahram et al., 2012).

During the last 30 to 40 years, several studies have been performed trying to clarify the possible relationship among the different species of *Plasmodium*. However, several discrepancies were found in these studies and several questions remain unclear.

Molineaux et al (1980) in a study conducted with Nigerian adults concluded that mixed infections were more common than expected and *P. malariae* is more commonly associated with *P. falciparum* than it was initially predictable based on the individual frequencies of each species. On the other hand, Richie (1988) and McKenzie & Bossard (1999), reported less mixed infections than would be expected when they compared *P. vivax – P. falciparum* infections, however high numbers of *P. malariae – P. falciparum* mixed infection were still found.

Another aspect that has been widely studied is the impact that mixed infections may have in the severity of the infection. Some studies reported a reduction in the severity of symptoms when mixed infections were present. In a study with African children, mixed infections of *P. falciparum* and *P. malariae* and / or *P. ovale* did not present any kind of symptoms, or if they existed were much less severe than *P. falciparum* simple infections (Mayxay et al., 2004). Similar results were obtained in a study in Vanuatu with children with α-thalassemia (Williams et al., 1996; Maitland et

al., 1997), where mixed infection of *P. falciparum* and *P. malariae* and / or *P. ovale* and *P. falciparum* + *P. vivax* presented a reduction in the severity of the symptoms. Luxemburger et al. (1997), in a study conducted in Thailand, showed a decrease of 5.7% to 1.6% on the severity of the infection when they compared single *P. falciparum* infection with *P. falciparum* + *P. vivax* infections. These results were corroborated by other studies carried out in different countries as Vanuatu (Williams et al., 1996), Papua New Guinea (Smith et al., 2001), Thailand (Price et al., 2001; McKenzie et al., 2006) and Brazil (Lorenzetti et al., 2008).

Depending on the relationship between parasites, it is possible to distinguish between positive and negative interactions. The first is characterized by the presence of a particular species favouring the development of another, while negative interactions are characterized by the presence of a species inhibiting the presence of another.

II.5. Parasite diversity

The extent of genetic diversity of natural populations of *Plasmodium* is enormous and both inter- and intra-specific infections are common. The understanding of this topic is a key epidemiological issue as ecological interactions between parasite populations in the same host may be an important source of selection on pathogen traits such as virulence and drug resistance and allows determining the influence of different parasite populations on infection and transmission dynamics. Further, some of those effects on infection parameters seem to be dependent on the seasonality and the intensity of malaria transmission (Marques et al., 2005).

II.5.1. Plasmodium falciparum

The genetic diversity of *P. falciparum* has been highly studied not only because this parasite is responsible for the highest number of clinical cases and deaths, but also due to its importance as an indicator of the malaria transmission intensity in an area (Paul et al., 1998); its ability to differentiate between recrudescence (which correspond to a treatment failure) and new infection (either from pre-existing liver infection or a newly established infection from an infected mosquito bite); its importance to the

development and/or the evaluation of malaria vaccines, since its high levels of genetic diversity is one of the biggest limitation for the development of an effective malaria vaccine (Schwartz et al., 2012).

The most frequently used markers for the *P. falciparum* genotyping are the merozoite surface protein 1 and 2 (MSP-1 and MSP-2) and the glutamate-rich protein (GLURP), which are surface antigens (Smythe et al., 1991; Snounou et al., 1998). With these markers it is impossible to know whether observed patterns reflect population history or natural selection (Anderson et al., 2000) so, nowadays, neutral microsatellite sequences (or short tandem repeats, STRs), are the most commonly used markers to differentiate *P. falciparum* populations.

Below, a brief description of the molecular markers used in this work is presented.

II.5.1.1. Genetic markers – msp2

The *msp2* gene codes for a merozoite surface antigen and is one of the most widely used genetic markers in population biology studies of *P. falciparum* parasites. This gene contains highly polymorphic regions with repeated units; in addition to size differences, it is possible to distinguish two allelic families – IC and FC27 (Smythe et al., 1991).

Through the analysis of *msp2* gene, it is possible to determine the multiplicity of infections (MOI), which is defined as the minimum number of different genotypes of *P. falciparum* in a single individual (Beck et al., 1997). MOI is a good indicator of acquired immunity or premunition in populations living in endemic areas and is also a good indicator of the malaria transmission intensity (Babiker et al., 1995; Paul et al., 1998). An area with high endemicity usually presents extensive parasite diversity and multiple genotypes are found in a single infected individual. In the opposite, the parasite population in a low transmission area shows limited genetic diversity and the majority of infections are monoclonal (Haddad et al., 1999; Babiker et al., 2001; Peyerl-Hoffmann et al., 2001; Gomez et al., 2002).

II.5.1.2. STRs

STRs are simple DNA sequence repeats composed by short motifs, usually with 6 or less bases, that are repeated in tandem (Queller et al., 1993). These STRs are found in all organisms and are widespread throughout the genome.

They are considered very useful molecular markers for population genetic analysis (Goldstein & Schlötterer, 1999, Anderson et al., 2000) and for genetic linkage mapping (McCollum et al., 2007), since they are very abundant, highly polymorphic, co-dominant and easy to score.

It is possible to differentiate between neutral STRs, i.e., not subjected to selection, allowing the analysis of the genetic variability without selection effect (McCollum et al., 2007); and STRs flanking resistance genes, that may reveal effects of selective sweep. From the analysis of these STRs it is possible to characterize the evolutionary origin of resistant alleles, check if new mutations arise in different geographic regions or share a common ancestor (Roper et al., 2003; Anderson & Roper, 2005).

II.5.2. Plasmodium vivax

Plasmodium vivax has been, until very recently, a neglected parasite. Many details of their biology, epidemiology and pathogenesis is unknown. Now it is known that this parasite is capable of causing severe manifestations of the disease, like cerebral malaria, renal failure, hepatic dysfunction and even death (Barcus et al., 2007; Kochar et al., 2009) and due to the appearance and spread of drug resistance in *P. vivax* (Baird & Hoffman, 2004), is essential to know its population structure and genetic diversity.

To study the diversity of infection within this parasite, the markers of choice are STRs or some surface antigen genes, such as circumsporozoite protein (CSP) and merozoite surface protein 3 alpha (MSP-3α).

To obtain accurate comparisons of genetic diversity of global *P. vivax* populations, a protocol for STRs genotyping must be standardized, as it happens with *P. falciparum* genotyping (Anderson et al., 1999).

II.5.2.1. Genetic markers – csp

There are three strains of *P. vivax* described so far - classic *P. vivax* (also called *P. vivax* VK 210), *P. vivax* VK 247 and *P. vivax*-like. The *csp* gene is commonly used to differentiate them. These three strains although morphologically similar, differ in the central portion of CSP protein, the most abundant polypeptide present at the sporozoite surface (Kappe et al., 2004).

The *csp* gene is highly polymorphic and presents a central repetitive domain that varies in sequence and length among *Plasmodium* spp. The *P. vivax* VK247 (described by Rosenberg et al., 1989) is characterized by having in the central portion of this gene a nonapeptide repeat unit, ANGA(G/D)(N/D)QPG, whereas the *P. vivax* classic (described by Arnot et al., 1985) parasite presents the nonapeptide repeat GDRA(A/D)GQPA. The strain *P. vivax*-like (Qari et al., 1993), is characterized by having a 11-mer repeat sequence, APGNQ(E/G)GGAA, in the central portion of the *csp* gene.

III. Human Host

III.1. Human factors associated with susceptibility / resistance to malaria

Several studies reported that malaria is one of the strongest known forces for evolutionary selection of the human genome, mainly in factors associated to erythrocytes, which play a key role in parasite life cycle (Lell et al., 1999; Parikh et al., 2004; Kwiatkowski, 2005). This is not surprising, since this disease has been highly prevalent through thousands of years, and even today is one of the most important causes of child mortality and morbidity worldwide (WHO, 2013).

This topic has been largely studied and genes that are involved in the structure and /or functionality of the erythrocytes are those with the highest number of genetic variants that have the ability to protect against the death from malaria or against the intensity of clinical symptoms (Williams, 2006). Among the most common and best characterized protective polymorphisms are some haemoglobinopathies, as

haemoglobin S (HbS) or sickle cell disease, which is associated with the production of structurally variant forms, and the α- and β-thalassemia, which causes a reduction in the production of normal α - or β -globin respectively; some enzymopathies, like glucose-6-phophate dehydrogenase (G6PD) deficiency, pyruvate kinase (PK) deficiency; the ABO system and the Duffy antigen. The alleles responsible for these changes, present very high frequencies in areas where malaria is or was highly prevalent.

The first association between a human genetic polymorphism and the decrease of the rates of malarial infection, was observed in 1946, in a study of inpatients at a regional hospital in Northern Rhodesia, now Zambia (Beet, 1946), where the carriers of sickle cell trait presented lower rates of infection when compared with nonsicklers patients. Later, Haldane (1949) proposed that the high frequencies of thalassemia around the shores of the Mediterranean Sea were a consequence of a selective advantage against *P. falciparum* malaria, and not a result of an exceptionally high mutation rate. This proposition was known as the "malaria hypothesis", which states that certain human genetic polymorphisms have been selected to high frequencies because they protect against some effects of malarial infections. Thereafter, several studies have demonstrated this same theory (Jepson et al., 1997; Cappadoro et al., 1998; Mackinnon et al., 2000; Weatherall & Clegg, 2002; Aidoo et al., 2002; Mackinnon et al., 2005; Min-Oo & Gros, 2005).

III.1.1. Duffy antigen

Plasmodium vivax, despite being the second most prevalent species of *Plasmodium* in the world, with 70 million to 300 million clinical cases pear year (Baird, 2007; Galinski & Barnwell, 2008; Mueller et al., 2009) is practically absent in central and western Africa, due to the majority of the population be Duffy negative (i.e. do not possess the Duffy antigen at erythrocyte surface) (Langhi & Bordin, 2006).

The Duffy antigen, also called Duffy antigen / receptor for chemokines (DARC), is a glycosylated membrane protein that is encoded by a gene located in the chromosome 1 and is more abundant on the surface of reticulocytes than on mature erythrocytes (Donahue et al., 1968; Dracopoli et al., 1991). Until very recently, DARC

was the unique receptor known required for the entry of *P. vivax* in the RBC and was the responsible for the protection against this parasite.

DARC-coding gene is polymorphic presenting multiple alleles, among them the codominant FY*A and FY*B which encode for the two main alleles – Fy^a and Fy^b. Four genotypes may result from the combination of the main alleles, Fy(a^+b^+), Fy(a^+b^-), Fy(a^-b^+), Fy(a^-b^-) (Tournamille et al., 1995; Castilho et al., 2004; Rowe et al., 2009). The first three originate a Duffy-positive phenotype, most prevalent in Asian and in Caucasian populations and the last one originates a Duffy-negative phenotype, most prevalent in African people, who are consequently (theoretically) resistant to *P. vivax* infection.

The Fy(a^-b^-) genotype results from a point mutation -33T>C in the promoter region of allele FY*B, in the GATA box region, which prevents the link with the transcription factor h-GATA1 (Tournamille et al., 1995; Castilho et al., 2004).

Several studies over the years, have demonstrated that the lack of Duffy antigen prevents the invasion of erythrocytes by *P. vivax*. Miller and collaborators (1975), observed that resistance to *P. vivax* was directly associated with the Duffy negative phenotype, while a study conducted by Barnwell and collaborators (1989) demonstrated *in vitro*, that merozoites of *P. vivax* are unable to invade RBCs that do not express the Duffy antigen. More recently, a study conducted in nine African countries, where 2 588 blood samples were analysed, found only one sample infected with *P. vivax* in a Duffy positive individual (Culleton et al., 2008). This study confirmed that this *Plasmodium* species is virtually absent of Africa. Another important aspect was found by Kasehagen et al. (2007) in a study conducted in Papua New Guinea. This study showed that not only the Duffy negative homozygous individuals are protected against *P. vivax* invasion, but also that the heterozygous individuals carriers of a new Duffy negative allele [Fy(A^+; A^-)], which shows a 50% decrease in the expression of Fy, are significantly more protected against *P. vivax* infection than homozygous individuals [Fy(A^+; A^+)]. Moreover, these individuals, when infected, have significantly lower parasitaemias when compared to normal individuals.

A surprising result was found in studies conducted in Brazil and in some countries of Africa, where Duffy negative individuals were infected with *P. vivax*. A study conducted in Kenya, with children considered for a case-control study of severe

malaria caused by *P. falciparum*, found children infected with *P. vivax* despite being Duffy negative (Ryan et al., 2006). Similar results were found in the Amazon region in Brazil and other locations in West Africa, such as Angola and Equatorial Guinea, where Duffy - negative individuals were found to be infected with *P. vivax*. (Cavasini et al., 2007a; Cavasini et al., 2007b, Mendes et al., 2011). A study that is being developed in Equatorial Guinea, found nine Duffy - negative individuals, containing the mutation -33T > C in the promoter region of the FY*B allele , located in the region of " GATA box" , infected with strains of *P. vivax* - *P. vivax* classic and *P. vivax* VK247 (Mendes et al, 2011) . These recent data suggested that *P. vivax* may be evolving, using alternative receptors to bind and invade erythrocytes.

IV. Mosquito vector

Human malarial protozoa are transmitted by mosquitoes of the genus *Anopheles*. There are 465 formally recognized *Anopheles* species, approximately 70 of which have the capacity of transmit human malaria parasites and of these, about 40 species are considered of major importance (Hay et al., 2010). This mosquito genus is the most and best studied one, mainly because of their impact on human health, once they are vectors of several diseases such as malaria and filariasis (Sinka, 2013).

According to Richards & Davies (1977), the mosquito vectors of malaria are classified as belonging to the *Animalia* kingdom, *Arthropoda* phylum, *Insecta* class, *Pterigota* subclass, *Diptera* order, *Nematocera* sub-order, *Culicidae* family, *Anophelinae* sub-family and *Anopheles* genus.

Figure 3 shows the distribution of the main malaria vectors around the world. It is possible to observe that malaria endemic areas present more than one mosquito species. In the areas of study – Angola, Equatorial Guinea and Guinea-Bissau – the main malaria vectors are *Anopheles gambiae s.l.* and *A. funestus* (Pålsson et al., 1998; Cano et al., 2006; WHO, 2013; Sinka, 2013), and as secondary *A. moucheti moucheti* and *A. carnavalei* (Cano et al., 2006).

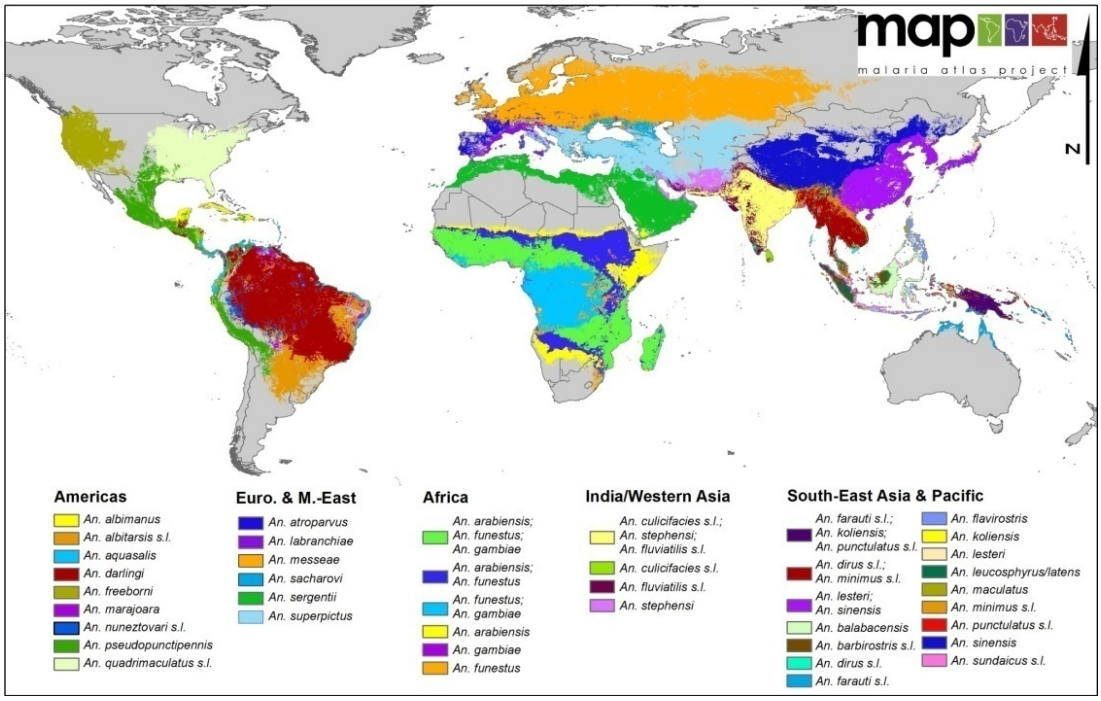

Figure 3. The global distribution of 34 dominant vector species and / or species complexes. (Reproduced from Sinka, 2013).

s.l.: *sensu lato* - meaning 'in the broad sense' referring to species complex.

IV.1. *Anopheles* life cycle

During their life-cycle, *Anopheles* mosquitoes, experience several stages of development: egg, larva, pupa and adult (Figure 4), being required two different habitats, an aquatic and a terrestrial habitat. The immature phases (egg, larva and pupa) are aquatic and have a duration between 5 and 14 days.

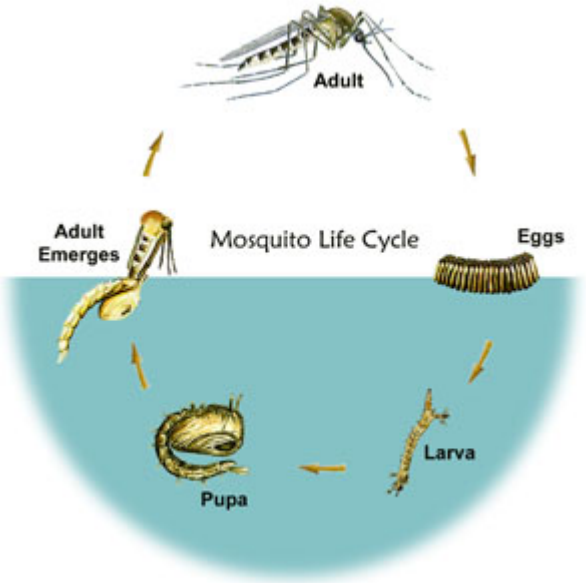

Figure 4. The life-cycle of *Anopheles* (adapted from http://www.epa.gov/pesticides/health/mosquitoes/mosquitojoint.htm, accessed in January 5th, 2013).

The duration of the complete mosquito's life-cycle depends on the species and temperature and may take between 7 to 21 days (Knell, 1991). The first activity of the new-hatched adult is the mating. The female only copulates once, because the last thing that the male does, is to inject a sealing substance which blocks the passage of other sperms. The *Anopheles* female can feed on nectar or fruit juices but to be able to produce eggs, she needs a blood meal. The female is capable of laying up to 50 to 200 eggs at once, and egg posture is done on the surface of the water. The egg takes between 1-2 days to hatch, originating the larva that go through four different stages. In the larval stage is possible to distinguish three main structures: the head, a broad and flattened thorax and a segmented abdomen. The larva lasts for approximately 7 days at tropical temperatures and for 2 to 4 weeks at lower temperatures. At the fourth stage, the larvae metamorphose into the pupa.

The pupal stage takes between 1 and 2 days; it does not feed but is mobile. In this stage, the pupa is comma-shaped with a distended cephalothorax and a segmented abdomen. After 2-3 days the adult emerge.

IV.2. Malaria vectors

Brief descriptions of the main *Anopheles* species present in the areas of study are presented below.

IV.2.1. Anopheles gambiae s.l.

The complex *Anopheles gambiae* includes seven sibling species that are morphologically indistinguishable, but distinguishable through their genetic and eco-ethological characteristics (della Torre et al., 2002). The seven members of these complex are: *A. gambiae* s.s. Giles, 1902; *Anopheles arabiensis* Paton, 1905; *Anopheles quadriannulatus* Theobald, 1911; *Anopheles quadriannulatus* B Hunt, 1998*; Anopheles melas* Theobald, 1903*; Anopheles merus* Donitz, 1902 e *Anopheles bwambae* White, 1985 (Scott et al., 1993). The *A. gambiae* s.s. and the *A. arabiensis* are considered the main and most effective vectors of malaria in sub-Saharan Africa, while *A. melas* and *A. merus* are considered secondary vectors. The distribution of *A. bwambae* is very restricted and, finally, *A. quadriannulatus* is not a malaria vector, since it is a zoophilic species. The others are mainly anthropophilic. These species also differ regarding the habitats: *A. gambiae s.s., A. arabiensis, A. quadriannulatus* are all freshwater breeding species; while *A. melas* and *A. merus* are salt water adapted species. *Anopheles bwambae* is a localised hot spring water adapted species. In 1998, a seventh member, *A. quadriannulatus* species B, has been proposed (Hunt et al., 1998), now called *Anopheles amharicus* Hunt (Coetzee et al., 2013).

Since individual species within the species complex differ in host-biting preference, abundance and vector competence, identification of the mosquito vectors to species level and mapping species distribution in heterogeneous environments are crucial to malaria epidemiology and control.

IV.2.2. Anopheles funestus

Like *A. gambiae* s.s., *A. funestus* s.s. is one of the major malaria vector in Africa. *Anopheles funestus* s.s. belongs to *A. funestus* Giles complex, which contains nine species, morphologically identical, that are distributed throughout Africa: *Anopheles*

parensis Gillies, *Anopheles aruni* Sobti, *Anopheles confusus* Evans and Leeson, *Anopheles funestus*, *Anopheles vaneedeni* Gilles and Coetzee, *Anopheles rivulorum* Leeson, *Anopheles fuscivenosus* Leeson, *Anopheles leesoni* Evans, and *Anopheles brucei* Service (Gillies & Coetzee, 1987). These sibling species present differences in their biology and vectorial competency, and the *A. funestus* s.s., is the only species of this complex that is anthropophilic.

The typical habitat for *A. funestus* larvae is a large, permanent or semi-permanent body of fresh water with emergent vegetation, like swamps and large ponds. This species is virtually present in all African continent because is very adaptable: it can be found in a wide range of altitudes [900m in Madagascar (Andrianaivolambo et al., 2010), 1400m in Central Africa (Tchuinkam et al., 2010) and up to 2000m in Kenya (Okaraet al., 2010)] and in a variety of breeding sites.

Anopheles funestus s.s. is highly anthropophilic and endophilic, which combined with a high longevity, makes it an efficient vector, as good as or better than *A. gambiae* in some areas. The remaining species of the complex are mainly zoophilic, but can occasionally feed on humans (Gilles & De Meillon, 1968). Some studies found *A. rivulorum* infected with *P. falciparum* in Tanzania (Wilkes et al., 1996; Temu et al., 2007), and Temu and collaborators (2007) also found positive specimens of *A. leesoni* and *A. parensis* with *P. falciparum*, suggesting a secondary role of these mosquitoes in malaria transmission.

IV.2.3. Anopheles carnevalei

Anopheles carnelavei belongs to the *Anopheles nili* group which includes four different species: *Anopheles nili s.s.*, *Anopheles somalicus*, *A. carnevalei* and *Anopheles ovengensis*. These species can be distinguished through slight morphologic characters observable at the larval and/or adult stages. Mosquitoes of this group are recognized as major human malaria vectors in tropical Africa, especially in areas with vegetation or dense shade along the rivers that represent typical larval development sites (Gillies & De Meillon, 1968).

Cano and collaborators (2003) described the presence of *A. carnevalei* in the continental region of Equatorial Guinea, mainly near rivers areas, and proved to have a great receptivity to the *Plasmodium* infection (Cano et al., 2006).

It is known that *A. carnevalei* is mostly zoophilic although it regularly feeds on humans in villages situated close to its breeding sites, and it is rarely collected resting indoors biting more frequently outdoors (Awono-Ambene et al., 2009). Despite its importance in terms of public health – it is an important malaria vector - this species is very poorly studied and little is known about their biology, ecology and genetics (Fontenille & Simard, 2004).

IV.2.4. Anopheles moucheti moucheti

Anopheles moucheti belongs to a group of three morphological forms: *A. moucheti moucheti*, *Anopheles moucheti bervoetsi* and *Anopheles moucheti nigeriensis,* distinguishable by slight morphological characters present at the adult and/or larval stages (Gillies & Coetzee, 1987; Fontenille & Simard, 2004).

In rural villages situated in deep forest areas, *A. moucheti* usually is the major vector of *Plasmodium* and quite often the only one maintaining a high level of malaria endemicity in humans (Shah et al., 2011).

Anopheles moucheti larvae are mainly found associated to floating vegetation of slow-moving streams or rivers and low temperatures (Gillies & De Meillon, 1968). *Anopheles moucheti* depends strongly on human blood, being highly anthropophilic, and tends to bite indoors (high densities of blood-fed females can be collected resting indoors). However, high mosquito densities might also be collected far from any human settlements, indicating a probable zoophilic behaviour in some forest populations (Antonio-Nkondjio & Simard, 2013).

IV.3. Mosquito immunity

During their life cycle, mosquitoes are exposed to a wide variety of pathogens and to cope with the risk of infection they have developed various defence mechanisms.

In fact, during the parasite population development in the mosquito three main bottlenecks are observed, which represent a large decrease in the number of parasites (Figure 5). The greatest reduction in parasite numbers occurs at the ookinete-to-oocyst transition stage. The ookinetes, are eliminated mainly by lysis or melanisation in the mosquito midgut epithelium and the ones that survives and reach the oocyst stage, multiply and produce thousands of sporozoites. When the oocysts burst, sporozoites are released to the hemolymph, invade the salivary glands and, upon subsequent mosquito bites, infect human hosts. It is estimated that in a blood meal, the mosquito ingest an average of 10 000 gametes and only 1 000 ookinetes develop successfully, and from those, less than five survives and develop to oocysts in mosquitoes.

The huge reduction in the number of parasites is explained by the capacity of the mosquito immune system trigger a series of mechanisms, limiting the *Plasmodium* infection (Blandin & Levashina, 2004; Whitten et al., 2006; Barrilas-Mury, 2007).

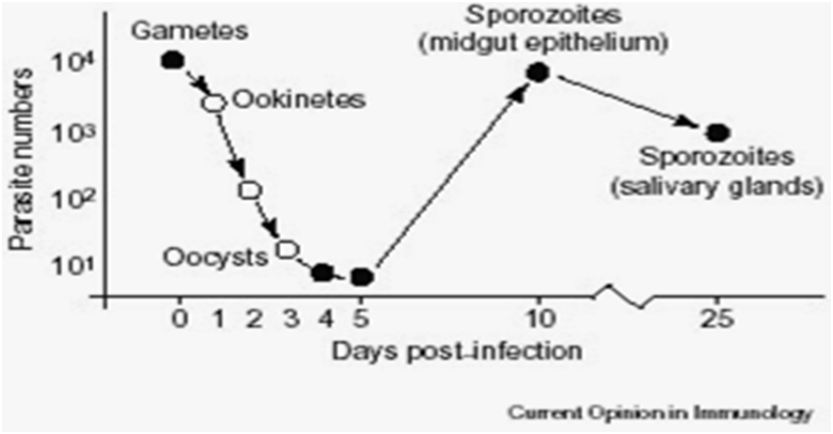

Figure 5: Parasite numbers during the critical steps of transformation of gametes to ookinetes of ookinetes to oocysts, and through the migration of sporozoites from the midgut epithelium to salivary glands (adapted from Blandin & Levashina, 2004).

These defence mechanisms involve not only structural barriers such as the rigid exoskeleton and peritrophic matrix (secreted after eating), as also a strong innate immune response against the parasite, including a wide variety of cellular and humoral mechanisms performed by various organs and cell types (Figure 6). This response

begins when pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptor (PRR) molecules (Dziarski, 2004). After this recognition, some reactions of the immune response will be triggered, including the activation of the cascade of serine proteases (proteolytic enzymes), which will regulate the activation of defence mechanisms as mosquito melanisation, synthesis of anti-microbial peptides or coagulation (Dimopoulos et al., 2001).

In *Anopheles* mosquitoes there are several families of genes that codify to PRRs, such as: peptidoglycan recognition proteins (PGRPs); gram-negative bacteria-binding proteins (GNBPs); thioester-containing proteins (TEPs); C-type lectins (CTLs); leucine-rich immune proteins (LRIMs); and scavenger receptors (SCRs) (Christophides et al., 2004; Michel & Kafatos, 2005; Osta et al., 2004; Yassine & Osta, 2010).

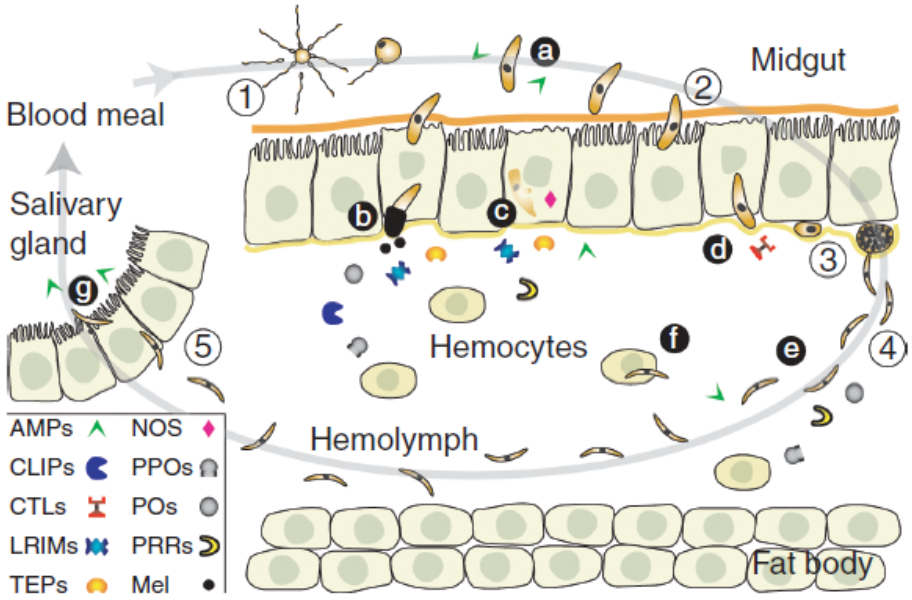

Figure 6. Schematic representation of the mechanisms of defence in *A. gambiae* against *Plasmodium* (adapted from Christophides et al., 2004).

1) Gametocyte activation and fertilization of gametes. It occurs soon after mosquito ingestion of parasite-infected blood meal. 2) Mosquito midgut invasion by ookinetes at approximately 1 day post infection. The invaded epithelial cells undergo apoptosis and are expelled from the epithelium into the midgut lumen. 3) Oocyst formation, a major parasite amplification step. Within the oocyst, repeated mitotic divisions give rise to thousands of sporozoites. 4) Oocyst rupture and sporozoite release in the mosquito haemocoel. The sporozoites migrate through the hemolymph toward the mosquito salivary glands. 5) Sporozoite invasion of the salivary glands. Following invasion, the parasites reside in the salivary gland lumen and during the next mosquito bite are injected into a new host. (a) Major losses are

documented during the first 24 h after infection. These losses possibly may be in part due to a mosquito immune reaction [e.g. secretion of antimicrobial peptides (AMPs)]. (b) Ookinete melanisation in a melanising refractory strain. Immune factors such as TEPs and LRIMs are involved in ookinete killing followed by melanisation, which depends on proteolytically activated prophenoloxidases [PPOs (POs)] and may be favoured also by elevated levels of reactive oxygen species. (c) A majority of ookinetes are lysed inside the cytoplasm of the midgut cells. Overproduction of nitric oxide produced by nitric oxide synthase (NOS) and other reactive species may lead to ookinete killing. (d) A number of ookinetes escape to the basal (haemocoel) side of the epithelium and evade immune responses by interacting with C-type lectins (CTLs). (e) Oocyst rupture and sporozoite release coincide with another immune response, possibly mounted by the mosquito fat body. (f) Phagocytosis and/or cellular melanotic encapsulation of *Plasmodium gallinaceum* sporozoites by hemolymph haemocytes has been observed in *Aedes aegypti*. (g) Induction of immune molecules is also observed during sporozoite invasion of the mosquito salivary glands.

PGRPs and *GNBPs* are some of the most studied PRRs. *PGRPs* are soluble or transmembrane proteins containing a domain similar to the bacterial amidase domain, which is involved in recycling bacterial cell wall fragments.

In *Drosophila*, the *PGRP-SA* activates the Toll pathway in response to Gram-positive bacterial infection together with *GNBP1*. On the other hand, *PGRP-LC* and *PGRP-LE* are involved in activating the immune deficiency (Imd) pathway in response to Gram-negative bacterial infections. Among the seven identified putative *Anopheles* PGRPs, PGRP-LC seems to play a central role in defence against bacterial infection (Osta et al., 2004).

GNBPs share significant sequence similarity with the catalytic region of bacterial β-1,3- and β-1,3,1,4-glucanases. In *A. gambiae*, there are six putative GNBPs described and GNBP-B1 and GNBP-A1 are up-regulated after *Plasmodium* infection, while only GNBP-B1 is reactive to bacteria (Dimopoulos et al., 2002).

TEP1 is a complement-like protein and is secreted into the hemolymph , where it is proteolytically matured by unknown proteases into two chains that remain associated together. TEP1 functions as an opsonin by binding covalently to the surface of Gram-negative and Gram-positive bacteria, in a thioester-dependent manner, triggering their clearance by phagocytosis. In refractory *A. gambiae* mosquitoes, the *knockout* of TEP1 results in an increase of *Plasmodium berghei* oocysts development, clearly showing its

antiparasitic effect. Similar results were found for *LRIM1* and *APL1C* genes (reviewed in Yassin & Osta, 2010).

Others PRRs, as *CTL4* and *CTLMA2*, have a completely different effect. The knockout of these two genes in the mosquito results in a drastic reduction in the number of oocysts formed, due to the melanisation of the ookinetes (Christophides et al., 2004; Michel & Kafatos, 2005).

IV.3.1. Signal modulation and transduction

In insects, the recognition of PAMPs by PRRs often triggers the activation of protease cascades that amplify the danger signal and relay it to downstream effector mechanisms promoting the elimination of the intruder. Between these effector mechanisms it is possible to highlight the coagulation, synthesis of antimicrobial peptides and melanisation.

The main components of these cascades are clip-domain serine proteases (CLIPs), that are involved is several defence mechanisms such as the activation of signalling pathways leading to the synthesis of antimicrobial peptides (AMPs), hemolymph agglutination and melanisation (Michel & Kafatos, 2005); and serpins, that are serine protease inhibitors (SRPNs) that sub-regulate or suppress the signal acting as suicide substrates of serine proteases (Christophides et al., 2004; Osta et al., 2004).

The identification of components of signalling pathways in the immune defence response of the mosquito was possible through the comparative analysis of the genomes of *Drosophila melanogaster* and *A. gambiae*. Two signal transduction pathways are described: the Toll and Imd pathways. The Toll pathway is activated by fungal or Gram-positive bacterial infections, inducing the proteolytic cleavage of Spaetzle, which binds directly to and activates the transmembrane receptor Toll; whereas the Imd pathway, is activated by Gram-negative bacteria, leading to the cleavage of a Rel/NFκB family protein, Relish, through the proteolytic action of the caspase Dredd (Christophides et al., 2002).

The majority of the intracellular components of the Toll and Imd pathways are conserved, and it is possible to find both in the mosquito and in *Drosophila*. However

there are few exceptions and the absence of the mosquito orthologous of *D. melanogaster* NF-kB transcription factor Dif is one of them. The mosquito genome encodes only two NF-kB transcription factors: Rel1 and Rel2, orthologous of *Drosophila* Drosal and Relish genes, respectively (Christophides et al., 2002).

These transcription factors, Rel1 and Rel2, regulates the levels of expression of some antiparasitic genes such as TEP1, and LRIM1 APL1C, interfering with protection against *Plasmodium* spp.

On the other side, the mosquito *A. gambiae* have two STAT (signal transducers and activators of transcription) genes that appear to have been originated by gene duplication (AgSTAT-A and AgSTAT-B). AgSTAT-B translocate to the nucleus of the fat body cells in response to bacterial challenge and regulates the levels of STAT-A. In fact, recent evidences stand out the role of STAT in the reduction of *P. berghei* and *P. falciparum* infections in the mosquito midgut (Yassine & Osta, 2010; Cirimotich et al., 2010).

IV.3.2. Effector mechanisms

IV.3.2.1. Antimicrobial peptides

At the final steps of the mosquito vector immune response, this triggers a series of effectors mechanisms, including the AMPs production. The AMPs are small effectors peptides, positively charged, highly abundant and diverse. They are produced systemically in the fat body and then secreted into the hemolymph, where they accumulate in high concentrations and spread throughout the entire body (Christophides et al., 2004; Michel & Kafatos, 2005).

Although several families of AMPs have been described in *Drosophila*, in most of the insects, two major families stand out: defensins and cecropins. In *A. gambiae* four families of AMPs have been identified: four defensins (Def), four cecropins (Cec), one attacin, and one gambicin (Gam) (Christophides et al., 2002). Def are cysteine-rich peptides and act mainly against Gram-positive bacteria, whereas the Cec are peptides with a α-helical structure more effective against Gram-negative bacteria or fungi (Christophides et al., 2004).

Several of these AMPS have shown antimicrobial activity: Gam and Cec1 have a broad spectrum of activity against Gram-type bacteria; Cec1 is active against yeasts and Gam is partially lethal in *in vitro* cultures of ookinetes of *P. berghei*. Def1 is mainly active against Gram-positive and some species of filamentous fungi. In *A. gambiae*, both Rel1 and Rel2 control the expression of Cec1, Gam and Def1 (Christophides et al. 2004; Osta et al., 2004; Michel & Kafatos, 2005; Cirimotich et al., 2010; Yassine & Osta, 2010).

IV.3.2.2. Melanisation

Melanisation is an immune defence response by which targeted microorganisms are involved in a layer of melanin. Melanisation is triggered when the recognition of microorganisms activates a CLIP cascade that culminates in the limited proteolysis and conversion of inactive prophenoloxidase (PPO) into active phenoloxidase (PO). Two tyrosine oxidation pathways, the dopa and dopamine pathways are associated with melanisation (Christophides et al., 2004; Yassine & Osta, 2010).

Parasite melanisation in the vector has been reported in many mosquito–parasite combinations but it seems to be dependent on the parasite species and strain. A well-known example is a refractory *A. gambiae* strain that melanises several *Plasmodium,* which occurs immediately after crossing of the midgut by the ookinete (Collins et al., 1986). Although these mosquitoes completely block the development of the primate malaria parasite *Plasmodium cynomolgi*, the rodent parasite *P. berghei*, and allopatric strains of the human parasite *P. falciparum*, fail to melanise its sympatric populations (Christophides et al., 2004; Yassine & Osta, 2010).

These results suggest that melanisation is not essential for defence against *Plasmodium* and it is also dispensable against bacterial infections, despite the fact that bacteria trigger PPO activation in the hemolymph (Schnitger et al., 2007).

IV.3.2.3. Phagocytosis

Another mechanism of cellular immune response in mosquitoes is the phagocytosis. This mechanism kills the microorganisms through the action of

haemocytes that recognize, surround and destroy pathogens and apoptotic cells. These actions are mediated by PRRs that bind to the particle and trigger intracellular cascades leading to its internalization through an actin dependent mechanism (Christophides et al., 2004; Yassine & Osta, 2010).

There are three types of haemocytes that have been characterized in adult mosquitoes: the oenocytoids, prohaemocytes and granulocytes which are the only phagocytic cells (Castillo et al., 2006).

In *A. gambiae*, the phagocytosis of *Escherichia coli* in an immune-competent cell line seems to be promoted by the TEP1, that binds to the bacterial surface through the thioester bond, similarly to human-complement factor C3 (Levashina et al., 2001).

IV.3.3. Coagulation

One of the key differences between vertebrates and arthropods is the fact that body fluids in vertebrates are mostly confined to blood and lymphatic vessels whereas arthropods have an open circulatory system. Because of that, arthropods had to find efficient mechanisms to prevent blood loss and also help in the trapping of microbes from entering and spreading throughout the hemocoel, after tissue damage. The hemolymph clotting is, therefore, an important mechanism of defence and involves both humoral and cellular responses (Dushay, 2009; Loof et al., 2011). The main actors in this process are the blood cells and soluble factors secreted by other organs such as the liver or the equivalent in insects, the fat body (Loof et al., 2011).

Clotting has been most studied in two non-insect arthropod species with significantly different clotting reactions: freshwater crayfish and the horseshoe crab. In crayfish, the clotting system depends on the direct transglutaminase (TG)-mediated cross-linking of a specific plasma protein, whereas in horseshoe crab the process is regulated by a proteolytic cascade, which is activated by bacterial elicitors through specific recognition proteins. Despite the differences between the distinct processes of clotting, in both invertebrates and vertebrates, the TGs stand out as the only component maintained in these processes (Theopold et al., 2004; Jiravanichpaisal et al., 2006).

IV.3.3.1. Transglutaminases

Transglutaminases (TGs; EC 2.3.2.13) are a family of structurally and functionally related enzymes that catalyse Ca^{2+}. They are widely distributed and have been identified in all organisms, from unicellular to mammals and plants. This family of enzymes is characterized by the lack of glycosylation and disulphide bonds, despite the presence of potential *N*-linked glycosylation sites and almost all TGs require calcium for the catalytic activity (Metha, 2005).

These enzymes are involved in numerous reactions, catalysing irreversible cross-linking of proteins forming isopeptide bonds between glutamine residues on one protein and primary amine groups on other proteins (Lorand & Corand, 1984); enable several cellular functions, such as cytoskeletal modifications and attachment to basement membrane (Bendixen et al., 1993); and are associated to the mediation of signal transduction pathways involved in apoptosis regulation (Im et al., 1997).

Transglutaminases structure

The structure of TG family is characterized by having four sequential and structurally distinct domains: an NH2-terminal β-sandwich, a α/β catalytic core, and two COOH-terminal β-barrel domains (Figure 7).

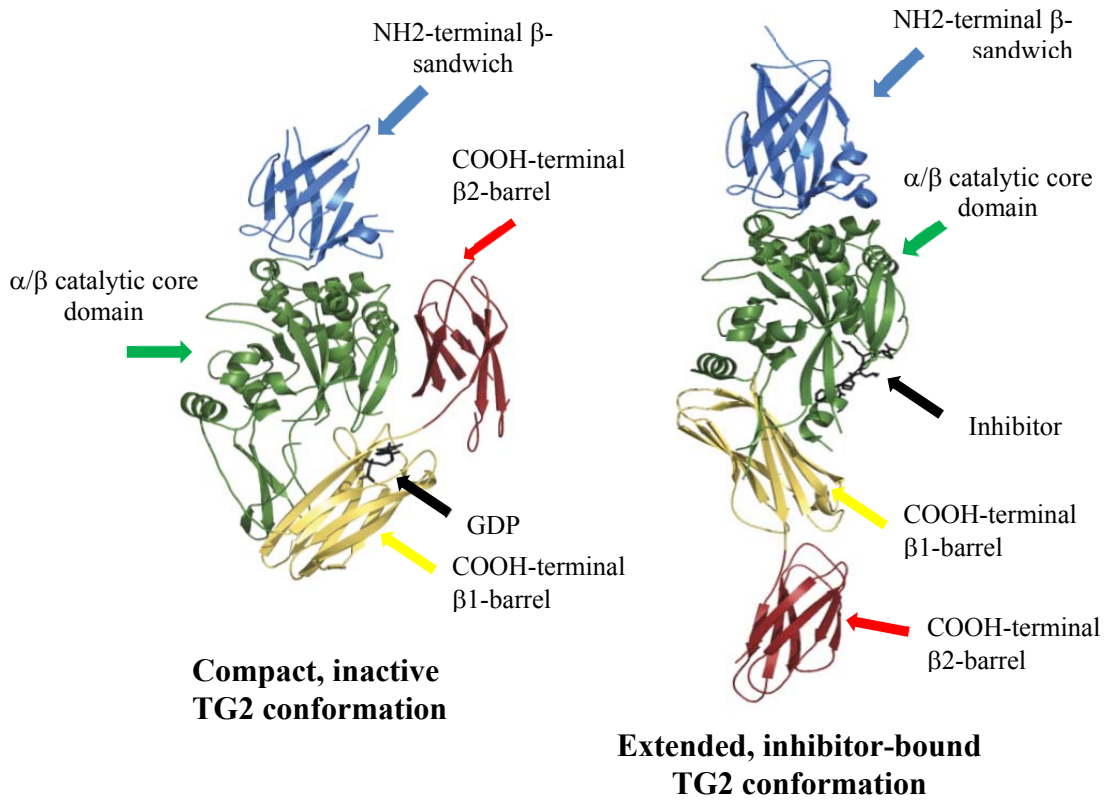

Figure 7. Transglutaminase tertiary structure, protein domains, and organization (adapted from Iismaa et al., 2009).

GDP and the reactive gluten-peptide mimic inhibitor Ac-P(DON)LPF-NH2 are shown as black lines.

The domain NH2-terminal β-sandwich consists of an initial flexible loop, the α/β catalytic core domain folds in two additional β-strands which move downwards and upwards along the surface of the core domain, containing the amino acids Ser^{171} and Lys^{173} that are involved in GTP (guanosine triphosphate) binding, and have four additional β-structures and four α-helices. The three first helices form a triangular arrangement forming the catalytic centre, and the forth helix is close to the very end of the core domain and contains the amino acids involved in the main Ca^{2+} binding region. Since the active centre of the enzyme is located deep in the α/β catalytic core domain, it isn't able to contact with substrates due to overlapping of the COOH-terminal β1 and 2-barrel domains (Beninati & Piacentini, 2004). Both of these domains are arranged as antiparallel β-barrels and are composed of six β strands and one β turn (COOH-terminal β1-barrel domains), and of seven antiparallel β-strands (COOH-terminal β2-barrel

domains). These C-terminal domains are important in regulating both transamidating activity and GTPase (and ATPase) activity (Griffin et al., 2002).

TGs present different conformations in presence / absent of Ca^{2+}, as shown in figure 7. In the absence of Ca^{2+} , the TG assume a compact conformation, with the domains NH2-terminal β-sandwich and the two COOH-terminal β-barrel bent forming structures-β, and the α/β domain presents a α-helical secondary structure (Griffin et al., 2002; Iismaa et al., 2009). On the other hand, in the presence of Ca^{2+} , the enzyme undergoes a conformational change and has an extended structure, becoming active (Griffin et al., 2002; Iismaa et al., 2009).

Distribution of Transglutaminases

Transglutaminases have been identified in several organisms as microorganisms, algae, plants, invertebrates, amphibians, fish, birds or mammals and in various tissues or body fluids.

In humans nine distinct TG were identified, but despite the overall primary structure appear to be different; they all share the same amino acid sequence at the active site. They are involved in different functions, as apoptosis, cell adhesion, and blood clotting, between other; and have a different distribution (Metha, 2005). Table 2 resumes the principal features of the nine human TG, which are common to the TG of mammals in general.

Table 2: Main characteristics of the nine human TG (Adapted from Metha, 2005).

Protein	Main functions	Distribution	Alternate names
TG1	Cell envelope formation during keratinocyte differentiation	Membrane-bound in keratinocytes	TG1,TG_k, keratinocyte TG, particulate TG
TG2	Apoptosis, cell adhesion, matrix stabilization, cell-survival signalling	Widely distributed in many tissues; cytosolic, nuclear, membrane, extracellular	Tissue TG, TG_c, liver TG, Gh_{α}, endothelial TG, erythrocyte TG,
TG3	Cell envelope formation during keratinocyte differentiation	Hair follicle, epidermis, brain	TG_E, callus TG, hair follicle TG, bovine snout TG
TG4	Reproduction especially in rodents as a result of semen coagulation	Prostate	Prostate TG, TG_P, androgen regulated major secretory protein, vesiculase, dorsal protein 1 (DP1)
TG5	Cornified cell envelope formation during keratinocytes differentiation	Foreskin keratinocytes, epithelial barrier lining and skeletal muscular striatum	TG_X
TG6	Not known	Testis and lung	TG_Y
TG7	Not known	Ubiquitous but predominantly in testis and lung	TG_Z
FXIIIa	Blood clotting, wound healing, bone growth	Platelets, placenta, synovial fluid, chondrocytes, astrocytes, macrophages	Fibrin-stabilizing factor, fibrinoligase, plasma TG, Laki-Lorand factor
Band 4.2	Major component in erythrocyte skeletal network	Erythrocyte membranes, bone marrow, spleen	B 4.2, ATP-binding erythrocyte membrane protein band 4.2

TGs were also described in crustaceans where these enzymes are mainly involved in the plasma clotting reaction (Hall et al., 1999; Wang et al., 2001). In the horseshoe crab, the TG is located in the amoebocytes and promotes the cross-linking of the cell surface proxin to coagulin, whereas in crayfish and in some species of shrimp, the TG is located in haemocytes and catalyses the cross-linking of the clotting protein (Yeh et al., 1999; Wang et al., 2001). Most recently, studies found that TG of horseshoe crab is involved in the host defence in the cuticle by cross-linking Caraxin-1, a component of cuticle, into a stable mesh, which promotes wound healing (Matsuda et al., 2007).

In *Drosophila* sp., TG is encoded by a single gene and is expressed both in haemocytes and in the fat body (Lindgren et al., 2008). This TG has an immune defence function. The immune response is almost instantaneous and starts when an intruder enters into contact with the hemolymph, leading to the formation of small aggregates, causing the sequestration of the intruders.

Anopheles gambiae mosquitoes have three genes encoding *TG1*, 2 and 3 (AGAP009100, AGAP009098 and AGAP009099, respectively) that are grouped in the chromosome 3R. The *AgTG3* is expressed exclusively in the male accessory glands (MAGs), while the other two are ubiquitous at a much lower levels. These results suggest that *AgTG3* is the main responsible for the TG activity detected in the MAGs, being together with the Plugin, responsible for the clot of the MAGs secretions (Rogers et al., 2009).

AgTG1 and *AgTG2* seems to be associated with coagulation/wound healing, however, little is known about its structure and location. Silveira et al. (2012) in a study where a TG inhibitor was injected in mosquitoes infected with *P. berghei,* showed an increasing in the rate of infection and in the oocyst load. These results suggest that the chemical inhibition of transglutaminase activity leads to significantly increased infection. Nsango et al. (2013) also showed that the *AgTG2* restricts development of human malaria parasite in *A. gambiae.*

References

Aidoo, M., Terlouw, D. J., Kolczak, M. S., McElroy, P. D., ter Kuile, F. O., Kariuki, S., Nahlen, B. L., Lal, A. A. & Udhayakumar, V. 2002. Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet*, 359, 1311-1312.

Alexandre, M. A., Ferreira, C. O., Siqueira, A. M., Magalhães, B. L., Mourão, M. P., Lacerda, M. V. & Alecrim, Md. 2010. Severe *Plasmodium vivax* Malaria, Brazilian Amazon. *Emerg Infect Dis*, 16, 1611-1614.

Anderson, T. J., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I. D., Brockman, A. H., Nosten, F., Ferreira, M. U. & Day, K. P. 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *P. falciparum*. *Mol Biol Evol*, 17, 1467-1482.

Anderson, T. J., Su, X. Z., Bockarie, M., Lagog, M. & Day, K. P. 1999. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. *Parasitology*, 119, 113-125.

Anderson, T. J.C. & Roper, C. 2005.The origins and spread of antimalarial drug resistance: Lessons for policy makers. *Acta Trop*, 94, 269-280.

Andrianaivolambo, L., Domarle, O., Randrianarivelojosia, M., Ratovonjato, J., Le Goff, G., Talman, A., Ariey, F. & Robert, V. 2010. Anthropophilic mosquitoes and malaria transmission in the eastern foothills of the central highlands of Madagascar. *Acta Trop*, 116, 240-245.

Antinori, S., Galimberti, L., Milazzo, L. & Corbellino, M. 2012. Biology of Human malaria plasmodia including *Plasmodium knowlesi*. *Mediterr J Hematol Infect Dis*. 4, e2012013.

Antonio-Nkondjio, C. & Simard, F. 2013. Highlights on *Anopheles nili* and *Anopheles moucheti*, Malaria Vectors in Africa. In: S. Manguin, ed. 2013. *Anopheles* mosquitoes – New insights into malaria. Croatia: InTech. Ch.4.

Arez, A. P., Pinto, J., Pålsson, K., Snounou, G., Jaenson, T. G. T. & do Rosário, V. E. 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum* genotypes. *Am J Trop Med Hyg*, 68, 161-168.

Arnot, D. E., Barnwell, J. W., Tam, J. P., Nussenzweig, V., Nussenzweig, R. & Enea, V. 1985. Circumsporozoite protein of *Plasmodium vivax* gene cloning and characterization of the immunodominant epitope. *Science*, 230, 815-818.

Awono-Ambene, P., Antonio-Nkondjio, C., Toto, J., Ndo, C., Etang, J., Fontenille, D. & Simard, F. 2009. Epidemological importance of the *Anopheles nili* group of malaria vectors in equatorial villages of Cameroon, Central Africa. *Sci Med Afr*, 1, 13-20.

Ayala, F. J.; Escalante, A. A.; Lal, A. A. & Rich, S. M. 1998. Evolutionary relationships of human malaria parasites in Malaria: parasite biology, pathogenesis and protection. *Sherman, I. W.* (ed.), 285-300.

Babiker, H. A., Charlwood, J. D., Smith, T. & Walliker, D. 1995. Gene flow and cross-mating in *Plasmodium falciparum* in households in a Tanzanian village. *Parasitology*, 111, 433-442.

Babiker, H. A., Lines, J., Hill, W.G. & Walliker, D. 1997. Population structure of *Plasmodium falciparum* in villages with different malaria endemicity in east Africa. *Am J Trop Med Hyg*, 56, 141-147.

Baird, J. K. 2007. Neglect of *Plasmodium vivax* malaria. *Trends Parasit*, 23, 533–539.

Baird, J. K., Hoffman, S. L. 2004. Primaquine therapy for malaria. *Clin Infect Dis*, 39, 1336-1345.

Barcus, M. J., Basri, H., Picarima, H., Manyakori, C., Sekartuti, Elyazar, I., Bangs, M. J., Maguire, J. D. & Baird, J. K. 2007. Demographic risk factors for severe and fatal *vivax* and *falciparum* malaria among hospital admissions in northeastern Indonesian Papua. *Am J Trop Med Hyg*, 77, 984-991.

Barillas-Mury, C., 2007. CLIP proteases and *Plasmodium* melanization in *Anopheles gambiae*. *Trends in Parasitol*, 23, 297-299.

Barnwell, J. W., Nichols, M. E. & Rubinstein, P. 1989. In vitro evaluation of the role of the Duffy blood group in erythrocyte invasion by *Plasmodium vivax. J Exp Med*, 169, 1795-1802.

Basco, L. K., Eldin de Pécoulas, P., Wilson, C. M., Le Bras, J. & Mazabraud, A. 1995a. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. *Mol Biochem Parasitol*, 69, 135-138.

Basco, L. K., Le Bras, J., Rhoades, Z. & Wilson, C. M. 1995b. Analysis of *pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from subsaharan Africa. *Mol Biochem Parasitol*, 74, 157-166.

Beck H. P., Felger I., Huber W., Steiger S., Smith T., Weiss N., Alonso P. & Tanner M. 1997. Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trial of the malaria vaccine SPf66. *J Infect Dis*, 175, 921-926.

Beet, E. A. 1946. Sickle cell disease in the Balovale district of Northern Rhodesia. *East Afr Med J*, 23, 75-86.

Belizario, V. Y., Pasay, C. J., Bersabe, M. J., de Leon, W. U., Guerrero, D. M. & Bugaoisan, V. M. 2005. Field evaluation of malaria rapid diagnostic tests for the diagnosis of *P. falciparum* and non-*P. falciparum* infections. *Southeast Asian J Trop Med Public Health*, 36, 552–561.

Bendixen, E., Borth, W. & Harpel, P. C. 1993. Transglutaminases catalyse cross-linking of plasminogen to fibronectin and human endothelial cells. *J Biol Chem*, 268, 21962-21967.

Beninati, S. & Piacentini, M., 2004. The transglutaminase family: an overview: minireview article. *Amino Acids*, 26, 367–372.

Blandin, S. & Levashina, E. A. 2004. Mosquito immune responses against malaria parasites. *Curr Opin Immunol*, 16, 16-20.

Bloland, P. B. 2001. Drug resistance in malaria. Geneve, Switzarland: World Health Organization.

Bousema, J. T., Drakeley, C. J., Mens, P. F., Arens, T., Houben, R., Omar, S. A., Gouagna, L. C., Schallig, H. & Sauerwein, R. W. 2008. Increased *Plasmodium falciparum* gametocyte production in mixed infections with ***P. malariae***. *Am J Trop Med Hyg*, 78, 442-448.

Bousema, T. & Drakeley C. 2011. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev*, 24, 377-410.

Bray, P. G., Martin, R. E., Tilley, L., Ward, S. A., Kirk, K. & Fidock, D. A. 2005. Defining the role of *PfCRT* in *Plasmodium falciparum* chloroquine resistance. *Mol Microbiol*, 56, 323-333.

Breman, J. G. 2001. The ears of the hippopotamus: manifestations, determinants and estimates of the malaria burden. *Am J Trop Med Hyg*, 64, 1-11.

Brooks, D. R., Wang, P., Read, M., Watkins, W. M., Sims, P. F. & Hyde, J. E. 1994 Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. *Eur J Biochem*, 224, 397-405.

Bruce, M. C., Donnelly, C. A., Alpers, M. P., Galinski, M. R., Barnwell, J. W., Walliker, D. & Day, K. P. 2000. Cross-species interactions between malaria parasites in Humans. *Science*, 287, 845-848.

Cano, J., Descalzo, M., Moreno, M., Chen, Z., Nzambo, S., Bobuakasi, L., Buatiche, J., Ondo, M., Micha, F. & Benito, A. 2006. Spatial variability in the density, distribution and vectorial capacity of anopheline species in a high transmission village (Equatorial Guinea). *Malar J*, 5: 21

Cano, J., Nzambo, S., Buatiche, J. N., Ondo-Esono, M., Micha, F. & Benito, A. 2003. *Anopheles* (Cellia) *carnevalei* in Equatorial Guinea (West-Central Africa). *J Am Mosq Assoc*, 19, 435-437.

Cappadoro, M., Giribaldi, G., O'Brien, E., Turrini, F., Mannu, F., Ulliers, D., Simula, G., Luzzatto, L. & Arese, P. 1998. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. *Blood*, 92, 2527-2534.

Carlton, J. M., Escalante, A. A., Neafsey, D. & Volkman, S. K. 2008. Comparative evolutionary genomics of human malaria parasites. *Trends Parasitol*, 24, 545–550.

Carter, R. & Mendis, K. N. 2002. Evolutionary and historical aspects of the burden of malaria. *Clin Microbiol Rev*, 15, 564-594.

Castilho, L., Rios, M., Pellegrinno, J. Jr., Saad, S. T. O., Costa, F. F. & Reid, M. E. 2004. A novel FY allele in Brazilians. *Vox Sang*, 87, 190-195.

Castillo, J.C., Robertson, A.E. & Strand, M.R. 2006. Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochem Mol Biol*, 36, 891-903.

Cavasini, C. E., de Mattos, L. C., Couto, A. A., Couto, V. S., Gollino, Y., Moretti, L. J., Bonini-Domingos, C. R., Rossit, A. R., Castilho, L. & Machado, R. L. 2007b. Duffy blood group gene polymorphisms among malaria *vivax* patients in four areas of the Brazilian Amazon region. *Malar J*, 6, 167-175.

Cavasini, C. E., Mattos, L. C., Couto, A. A., Bonini-Domingos, C. R., Valencia, S. H., Neiras, W. C., Alves, R. T., Rossit, A. R., Castilho, L. & Machado, R. L. 2007a. *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? *Trans R Soc Trop Med Hyg*, 101, 1042-1044.

Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P. T., Collins, F. H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N. T., Hoffmann, J. A., Kanzok, S. M., Letunic, I., Levashina, E. A., Loukeris, T. G., Lycett, G., Meister, S., Michel, K., Moita, L. F., Müller, H. M., Osta, M. A., Paskewitz, S. M., Reichhart, J. M., Rzhetsky, A., Troxler, L., Vernick, K. D., Vlachou, D., Volz, J., von Mering, C., Xu, J., Zheng, L., Bork, P. & Kafatos, F. C. 2002. Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298, 159-165.

Christophides, G. K., Vlachou, D. & Kafatos, F. C. 2004. Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. *Immunol Rev*, 198, 127-148.

Cirimotich, C. M., Dong, Y., Garver, L. S., Sim, S. & Dimopoulos, D. 2010. Mosquito immune defences against *Plasmodium* infection. *Dev Comp Immunol*, 34, 387–395.

Clyde, D. 1954. Observations on monthly pyrimethamine (‘Daraprim’) prophylaxis in an East African village. *East Afr Med J*, 3, 41–46.

Coetzee, M., Hunt, R. H., Wilkerson, R., Torre, A. D., Coulibaly, M. B. &Besansky, N. J. 2013. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. *Zootaxa*, 3619, 246–274

Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., Collins, W. E., Campbell, C. C. & Gwadz, R. W. 1986. Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science*, 234, 607–610.

Collins, W. E. & Jeffery, G. M. 2007. *Plasmodium malariae*: parasite and disease. *Clin Microbial Rev*, 20, 579-592.

Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. & Foote, S. J. 1988. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. *Proc Natl Acad Sci USA*, 85, 9109-9113.

Culleton, R. L., Mita, T., Ndounga, M., Unger, H., Cravo, P. V., Paganotti, G. M., Takahashi, N., Kaneko, A., Eto, H., Tinto, H., Karema, C., D'Alessandro,

U., do Rosário, V., Kobayakawa, T., Ntoumi, F., Carter, R. & Tanabe, K. 2008. Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing. *Malar J*, 7, 174.

Curtis, J., Duraisingh, M. T., Trigg, J. K., Mbwana, H., Warhurst, D. C. & Curtis, C. F. 1996. Direct evidence that asparagine at position 108 of the *Plasmodium falciparum* dihydrofolate reductase is involved in resistance to antifolate drugs in Tanzania. *Trans R Soc Trop Med Hyg*, 90, 678-680.

della Torre, A., Costantini, C., Besansky, N.J., Caccone, A, Petrarca, V., Powell, J.R. & Coluzzi, M. 2002. Speciation within *Anopheles gambiae* – the Glass Is Half Full. *Science*; 298, 115-117.

Dimopoulos, G., Christophides, G. K., Meister, S., Schultz, J., White, K. P., Barillas-Mury, C. & Kafatos, F. C. 2002. Genome expression analysis of *Anopheles gambiae*, responses to injury, bacterial challenge, and malaria infection. *Proc Natl Acad Sci USA*, 99, 8814-8819.

Dimopoulos, G., Müller, H. M., Levashina, E. A. & Kafatos, F. C. 2001. Innate immune defence against malaria infection in the mosquito. *Curr Opin Immunol*, 13, 79-88.

Donahue, R. P., Bias, W. B., Renwick, J. H. & McKusick, V. A. 1968. Probable assignment of the Duffy blood group locus to chromosome 1 in man. *Proc Natl Acad Sci USA*, 61, 949–955.

Dorn, A., Vippagunta, S. R., Matile, H., Jaquet, C., Vennerstrom, J. L. & Ridley, R. G. 1998. An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerisation by quinoline antimalarials. *Biochem Pharmacol*, 55, 727-736.

Dracopoli NC, O'Connell P, Elsner TI, Lalouel JM, White RL, Buetow, K. H., Nishimura, D. Y., Murray, J. C., Helms, C. & Mishra, S. K. 1991. The CEPH consortium linkage map of human chromosome 1. *Genomics*, 9, 686-700.

Dushay, M. S., 2009. Insect hemolymph clotting. *Cell Mol Life Sci*, 66, 643-2650.

Dziarski, R. 2004. Peptidoglycan recognition proteins (PGRPs). *Mol Immunol*, 40, 877-886.

Eisele, T. P., Larsen, D. A., Anglewicz, P. A., Keating, J., Yukich, J., Bennett, A., Hutchinson, P. & Steketee, R. W. 2012. Malaria prevention in pregnancy, birthweight, and neonatal mortality: a meta-analysis of 32 national cross-sectional datasets in Africa. *Lancet Infect Dis*, 12, 942-949.

Escalante, A. A. & Ayala, F.J. 1994. Phylogeny of the malaria genus *Plasmodium*, derived from rRNA gene sequence. *Proc Natl Acad Sci Usa*, 91, 11373-11377.

Escalante, A. A., Barrio, E. & Ayala, F.J. 1995. Evolutionary origin of human and primate malarias: evidence form the circumsporozoite protein gene. *Mol Biol Evol*, 12, 616-626.

Fontenille, D. & Simard, F. 2004. Unravelling complexities in human malaria transmission dynamicsin Africa through a comprehensive knowledge of vector populations. *Comp Immun Microbiol Infect Dis*, 27, 357 – 375.

Galinski, M. R. & Barnwell, J. W. 2008. *Plasmodium vivax*: who cares? *Malar J*, 7, S9.

Gamble, C. L., Ekwaru, J. P. & ter Kuile, F. O. 2009. Insecticide-treated nets for preventing malaria in pregnancy. *Cochrane Database Syst Rev*, 12, CD003755.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M, Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. & Barrell B. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*, 419, 498-451.

Gautret, P., Legros, F., Koulmann, P., Rodier, M. H. & Jacquemin, J. L. 2001. Imported *Plasmodium vivax* malaria in France: geographical origin and report of an atypical case acquired in Central or Western Africa. *Acta Trop*, 78, 177-181.

Genton, B., D'Acremont, V., Rare, L., Baea, K., Reeder, J. C., Alpers, M. P. & Müller, I. 2008. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: A prospective cohort study from Papua New Guinea. *PLoS Med*, 5, 881-889.

Gething, P. W., Elyazar, I. R. , Moyes, C. L., Smith, D. L., Battle, K. E., Guerra, C. A., Patil, A. P., Tatem, A. J., Howes, R. E., Myers, M. F., George, D. B., Horby, P., Wertheim, H. F., Price, R. N., Müeller, I., Baird, J. K. & Hay, S. I. 2012. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. *PLoS Negl Trop Dis*, 6, e1814.

Gilles, H. M. 1993. The malaria parasites. In: Gilles HM, Warrell DA (Eds). Bruce-Chwatt's Essential Malariology. 3rd Edition, London, Edward Arnold, 12-34.

Gillies, M. T. & Coetzee, M. T. 1987. A Supplement to the Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region). *S Afr Inst Med Res*, 2, 55.

Gillies, M. T. & de Meillon, B. 1968. The anophelinae of Africa, south of the Sahara. Johannesburg. *S Afr Inst Med Res*.

Goldstein, D. B. & Schlötterer, C. 1999. Microsatelites: Evolution and Application 1st edition. U.S.A. OXFORD University Press.

Gomez, D., Chaparro, J., Rubiano, C., Rojas, M. O. & Wasserman, M. 2002. Genetic diversity of *Plasmodium falciparum* field samples from an isolated Colombian village. *Am J Trop Med Hyg*, 67, 611-616.

Griffin, M., Casadio, R. & Bergamini, C. M., 2002. Transglutamninases: nature's biological glues. *Biochem J*, 368, 377-396.

Guerra, C. A., Howes, R. E., Patil, A. P., Gething, P. W., Van Boeckel, T.P., Temperley, W. H., Kabaria, C. W., Tatem, A. J., Manh, B. H., Elyazar, I. R., Baird, J. K., Snow, R. W. & Hay, S. I. 2010. The International Limits and Population at Risk of *Plasmodium vivax* Transmission in 2009. *PLoS Negl Trop Dis*, 4, e774.

Haddad, D., Snounou, G., Mattei, D., Enamorado, I. G., Figueroa, J., Stahl, S. & Berzins, K. 1999. Limited genetic diversity of *Plasmodium falciparum* in field isolates from Honduras. *Am J Trop Med Hyg,* 60, 30-34.

Haldane, J. B. S. 1948. The rate of mutation of human genes. *Hereditas* 35, 267-273.

Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen, L. & Söderhäll, K. 1999. The crayfish plasma clotting protein: A vitellogenin-related protein responsible for clot formation in crustacean blood. *Proc Natl Acad Sci USA*, 96, 1965-1970.

Hay, S. I., Sinka, M. E., Okara, R. M., Kabaria, C. W., Mbithi, P. M., Tago, C. C., Benz, D., Gething, P. W., Howes, R. E., Patil, A. P., Temperley, W. H., Bangs, M. J., Chareonviriyaphap, T., Elyazar, I. R., Harbach, R. E., Hemingway, J., Manguin, S., Mbogo, C. M., Rubio-Palis, Y. & Godfray, H. C. 2010. Developing global maps of the dominant anopheles vectors of human malaria *PLoS Med*, 7, e1000209.

Holmes, E. C. 2010. Malaria: the gorilla connection. *Nature,* 467, 404-405.

Hunt, R.H., Coetzee, M. & Fettene M. 1998. The *Anopheles gambiae* complex: a new species from Ethiopia. *Trans R Soc Trop Med Hyg,* 92, 231-235.

Iismaa, S. E., Mearns, B. M., Lorand, L. & Graham, R. M., 2009. Transglutaminases and disease: Lessons from genetically engineered mouse models and inherited disorders. *Physiol Rev*, 89, 991-1023.

Im, M. J., Russell, M. A. & Feng, J. F. 1997. Transglutaminase II: a new class of GTP-binding protein with new biological functions. *Cell Signal*, 9, 477-482.

Jeffares, D. C., Pain, A., Berry, A., Cox, A. V., Stalker, J., Ingle, C. E., Thomas, A., Quail, M. A., Siebenthall, K., Uhlemann, A. C., Kyes, S., Krishna, S., Newbold, C., Dermitzakis, E. T. & Berriman, M. 2006. Genome variation and evolution of the malaria parasite *Plasmodium falciparum*. *Nature Genetics*, 39, 120-125.

Jepson, A., Sisay-Joof, F., Banya, W., Hassan-King, M., Frodsham, A., Bennett, S., Hill, A.V. & Whittle, H. 1997. Genetic linkage of mild malaria to the major histocompatibility complex in Gambian children: study of affected sibling pairs. *BMJ*, 315, 96-97.

Jiravanichpaisal, P., Lee, B. L. & Söderhäll, K. 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immun*, 211, 213-236.

Kappe, S. H., Buscaglia, C. A. & Nussenzweig, V. 2004. *Plasmodium* sporozoite molecular cell biology. *Annu Rev Cell Dev Biol*, 20, 29-59.

Kasehagen, L. J., Mueller, I., Kiniboro, B., Bockarie, M. J., Reeder, J. C., Kazura, J. W., Kastens, W., McNamara, D. T., King, C. H., Whalen, C. C. & Zimmerman, P. A. 2007. Reduced *Plasmodium vivax* erythrocyte infection in PNG Duffy-negative heterozygotes. *PLoS One*, 2, e336.

Kleinschmidt, I., Schwabe, C., Benavente, L., Torrez, M., Ridl, F. C., Segura, J. L., Ehmer, P. & Nchama, G. N. 2009. Marked increase in child survival after four years of intensive malaria control. *Am J Trop Med Hyg*, 80, 882-888.

Knell, A. J. 1991. Malaria. A publication of the Tropical programme of the Wellcome Trust. *Oxford University Press*. 89pp

Kochar, D. K., Das, A., Kochar, S. K., Saxena, V., Sirohi, P., Garg, S., Kochar, A., Khatri, M. P. & Gupta, V. 2009. Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. *Am J Trop Med Hyg*, 80, 194-198.

Kublin, J. G., Dzinjalamala, F. K., Kamwendo, D. D., Malkin, E. M., Cortese, J. F, Martino, L. M., Mukadam, R. A., Rogerson, S. J., Lescano, A. G., Molyneux, M. E., Winstanley, P. A., Chimpeni, P., Taylor, T. E. & Plowe, C. V. 2002. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis*, 185, 380-388.

Kwiatkowski, D. P. 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet*, 77, 171-192.

Kyabayinze, D. J., Tibenderana, J. K., Odong, G. W., Rwakimari, J. B. & Counihan, H. 2008. Operational accuracy and comparative persistent antigenicity of HRP2 rapid diagnostic tests for *Plasmodium falciparum* malaria in a hyperendemic region of Uganda. *Malar J*, 7, 221.

Langhi, D. M. Jr. & Bordin, J. O. 2006. Duffy blood group and malaria. *Hematology*. 11, 389-398.

Le Bras, J. & Durand, R. 2003. The mechanisms of resistance to antimalarial drugs in *Plasmodium falciparum*. *Fundam Clin Pharmacol*, 17, 147-153.

Lell, B., May, J., Schmidt-Ott, R. J., Lehman, L. G., Luckner, D., Greve, B., Matousek, P., Schmid, D., Herbich, K., Mockenhaupt, F. P., Meyer, C. G., Bienzle, U. & Kremsner, P. G. 1999. The role of red blood cell polymorphisms in resistance and susceptibility to malaria. *Clin Infect Dis*, 8, 794-799.

Levashina, E. A., Moita, L. F., Blandin, S., Vriend, G., Lagueux, M. & Kafatos, F. C. 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell*, 104, 709-718.

Lindgren, M., Riazi, R., Lesch C., Wilhelmsson C., Theopold U. & Dushay, M.S., 2008. Fondue and transglutaminase in the *Drosophila* larval clot. *J Insect Physiol*, 54, 586-592.

Liu, W., Li, Y., Learn, G. H., Rudicell, R. S., Robertson, J. D., Keele, B. F., Ndjango, J. B., Sanz, C. M., Morgan, D. B., Locatelli, S., Gonder, M. K., Kranzusch, P. J., Walsh, P. D., Delaporte, E., Mpoudi-Ngole, E., Georgiev, A. V., Muller, M. N., Shaw, G. M., Peeters, M., Sharp, P. M., Rayner, J. C. & Hahn, B. H. 2010. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. *Nature*, 467, 420-425.

Loof, T. G., Schmidt, O., Herwald H. & Theopold U., 2011. Coagulation systems of invertebrates and vertebrates and their roles in innate immunity: The same side of two coins? *J Innate Immun*, 3, 34-40.

Lorand, L. & Conrad, S. M. 1984. Transglutaminases. *Mol Cell Biochem*, 58, 9-35.

Lorenzetti, A., Fornazari, P. A., Bonini-Domingos, A. C., de Souza Rodrigues Penhalbel, R., Fugikaha, E., Bonini-Domingos, C. R., Fraga, V. D., Conceição, L. M., Rossit, A. R., Cavasini, C. E., Couto, V. S. & Machado, R. L. 2008. Mixed *Plasmodium falciparum* infections and its clinical implications in four areas of the Brazilian Amazon region. *Acta Trop*, 107, 8-12.

Luxemburger, C., Ricci, F., Nosten, F., Raimond, D., Bathet, S. & White, N. J. 1997. The epidemiology of severe malaria in an area of low transmission in Thailand. *Trans. R. Soc. Trop. Med. Hyg*, 91, 256-262.

Mackinnon, M. J., Gunawardena, D. M., Rajakaruna, J., Weerasingha, S., Mendis, K. N. & Carter, R. 2000. Quantifying genetic and nongenetic contributions to malarial infection in a Sri Lankan population. *Proc Natl Acad Sci USA*, 97, 12661-12666.

Mackinnon, M. J., Mwangi, T. W., Snow, R. W., Marsh, K. & Williams, T. N. 2005. Heritability of malaria in Africa. *PloS Med*, 2, e340.

Maitland, K., Williams, T. N. & Newbold C. I. 1997. *Plasmodium vivax* and *P. falciparum*: biological interactions and the possibility of cross-species immunity. *Parasitol Today*, 13, 227-231.

Marques, P. X., Saúte, F., Pinto, V. V., Cardoso, S., Pinto, J., Alonso, P. L., do Rosário, V. E. & Arez, A. P. 2005. *Plasmodium* species mixed infections in two ecologically different areas of Manhiça District, Mozambique. *Int J Biol Sci*, 1, 96-102.

Mason, D. P. & McKenzie, E. 1999. Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax-Plasmodium falciparum* infections. *Am J Trop Med Hyg*, 61, 367-374.

Matsuda, Y., Koshiba, T., Osaki, T., Suyama, H., Arisaka, F., Toh, Y. & Kawabata, S. 2007. An arthropod cuticular chitin-binding protein endows injured sites with transglutaminase-dependent mesh. *J Biol Chem*, 282, 37316-37324.

Mayxay, M., Pukrittayakamee, S., Newton, P. N. & White, N. J. 2004. Mixed-species malaria infections in humans. *Trends in Parasitol*, 20, 233-240.

McCollum, A. M., Mueller, K., Villegas, L., Udhayakumar, V. & Escalante, A. A. 2007. Common origin and fixation of *Plasmodium falciparum* dhfr and dhps mutations associated with Sulfadoxine-Pyrimethamine resistance in a low-transmission area in South America. *Antimicrob Agents Chemother*, 51, 2085-2091.

Mckenzie, F. E & Bossert, W. H. 1999. Multi-species *Plasmodium* infections of humans. *J Parasitol*, 85, 12-18.

McKenzie, F. E., Smith, D. L., O'Meara, W. P., Forney, J. R., Magill, A. J., Permpanich, B., Erhart, L. M., Sirichaisinthop, J., Wongsrichanalai, C. & Gasser, R. A. Jr. 2006. Fever in patients with mixed-species malaria. *Clin Infect Dis*, 42, 1713-1718.

Menard, D., Barnadas, C., Bouchier, C., Henry-Malldin , C., Gray, L. R., Ratsimbasoa, A., Thonier, V., Carod, J. F., Domarle, O., Collin, Y., Bertrand, O., Picot, J., King, C. L., Grimberg, B. T., Mercereau - Puijalon, O. & Zimmerman, P. A. 2010. *Plasmodium vivax* clinical malaria is commonly

observed in Duffy-negative Malagasy people. *Proc Natl Acad Sci USA*, 107, 5967-5971.

Mendes, C., Dias, F., Figueiredo, J., Mora, V. G., Cano, J., de Sousa, B., do Rosário, V. E., Benito, A., Berzosa, P. & Arez, A. P. 2011. Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis*, 5(6), e1192.

Metha, K. 2005. Mammalian Transglutaminases: A Family Portrait. In: Mehta, K., Eckert, R. eds. 2005. Transglutaminases. Basel, *Karger, Prog Exp Tum Res,* 38, 1-18.

Michel, K. & Kafatos, F. C. 2005. Mosquito immunity against *Plasmodium*. *Insect Biochem Mol Biol*, 35, 677-689.

Miller, L. H., Mason, S. J., Dvorak, J. A., McGinniss, M. H. & Rothman, I. K. 1975. Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science*, 189, 561-563.

Min-Oo, G. & Gros, P. 2005. Erythrocyte variants and the nature of their malaria protective effect. *Cell Microbiol*, 7, 753-763.

Molina, R., Benito, A., Roche, J., Blanca, F., Amele, C., Sanchez, A., & Alvar, J. 1993. Baseline entomological data for a pilot malaria control programme in Equatorial Guinea. *J Med Entomol*, 3, 622-624.

Molineaux, L., Storey, J., Cohen, J. E. & Thomas, A. 1980. A longitudinal study of human malaria in the West African Savanna in the absence of control measures: relationships between different *Plasmodium* species, in particular *P. falciparum* and *P. malariae*. *Am J Trop Med Hyg*, 29, 725-737.

Moreno, M., Cano, J., Nzambo, S., Bobuakasi, L., Buatiche, J. N., Ondo, M., Micha, F. & Benito, A. 2004. Malaria Panel assay vs PCR: detection of naturally infected *Anopheles melas* in a coastal village of Equatorial Guinea. *Malar J*, 3, 20.

Mueller, I., Zimmerman, P. A. & Reeder, J. C. 2007. *Plasmodium malariae and Plasmodium ovale* - the "bashful" malaria parasites. *Trends Parasitol*, 23, 278-282.

Mueller, I., Galinski, M. R., Baird, J. K., Carlton, J. M., Kochar, D. K., Alonso, P. L. & del Portillo, H. A. 2009. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis*, 9, 555-566.

Murray, C. J. L., Rosenfeld, L. C., Lim, S. S., Andrews, K. G., Foreman, K. J., Haring, D., Fullman, N., Naghavi, M., Lozano, R. & Lopez, A. D. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *The Lancet*, 379, 413-431.

Nsango, S. E., Pompon, J., Xie, T., Rademacher, A., Fraiture, M., Thoma, M., Awono-Ambene, P. H., Moyou, R. S., Morlais, I. & Levashina, E. A. 2013. AP-1/Fos-TGase2 axis mediates wounding-induced *Plasmodium* killing in *Anopheles gambiae. J Biol Chem,* 288, 16145-16154.

Okara, R. M., Sinka, M. E., Minakawa, N., Mbogo, C. M., Hay, S. I. & Snow, R. W. 2010. Distribution of the main malaria vectors in Kenya. *Malar J*, 9, 69.

Osta, M. A., Christophides, G. K., Vlachou, D. & Kafatos, F. C. 2004. Innate immunity in the malaria vector *Anopheles gambiae*: comparative and functional genomics. *J Exp Biol*, 207, 2551-2563.

Pålsson, K., Pinto, J., do Rosário, V. E. & Jaenson, T. G. T. 1998. The palpal ratio method compared with PCR to distinguish between *Anopheles gambiae* s.s. and *A. melas* from Guinea Bissau. *Acta Trop*, 70, 101-107.

Pardo, G., Descalzo, M. A., Molina, L., Custodio, E., Lwanga, M., Mangue, C., Obono, J., Nchama, A., Roche, J., Benito, A. & Cano, J. 2006. Impact of different strategies to control *Plasmodium* infection and anaemia on the island of Bioko (Equatorial Guinea). *Malar J*, 5, 10.

Parikh, S., Dorsey, G. & Rosenthal, P. J. 2004. Host polymorphisms and the incidence of malaria in Ugandan children. *Am J Trop Med Hyg*,71, 750-753.

Pasvol, G., 2010. Protective hemoglobinopathies and *Plasmodium falciparum* transmission. *Nat Genet*, 42, 284–285.

Paul, R. E., Hackford, I., Brockman, A., Muller-Graf, C., Price, R., Luxemburger, C., White, N. J., Nosten, F. & Day, K. P.1998. Transmission intensity and *Plasmodium falciparum* diversity on the northwesternborder of Thailand. *Am J Trop Med Hyg*, 58, 195-203.

Payne, D. 1987. Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitol Today*, 3, 241-246.

Peyerl-Hoffmann, G., Jelinek, T., Kilian, A., Kabagambe, G., Metzger, W. G. & von Sonnenburg, F. 2001. Genetic diversity of *Plasmodium falciparum* and its relationship to parasite density in an area with different malaria endemicities in West Uganda. *Trop Med Int Health*, 6, 607-613.

Poirriez, J., Landau, I., Verhaeghe, A., Savage, A. & Dei-Cas, E. 1991. Atypical forms of *Plasmodium vivax*. Apropos of a case. *Ann Parasitol Hum Comp*, 66, 149-154.

President's Malaria Initiative, Angola, Malaria Operational Plan FY 2013. USAID (http://www.pmi.gov/docs/default-source/default-document-library/country-profiles/angola_profile.pdf?sfvrsn=22, accessed in October 20th, 2013).

Price, R. N., Simpson, J. A., Nosten, F., Luxemburger, C., Hkirjaroen, L., ter Kuile, F., Chongsuphajaisiddhi, T. & White, N. J. 2001. Factors contributing to anemia after uncomplicated *falciparum* malaria. *Am J Trop Med Hyg*, 65, 614-622.

Qari, S. H., Shi, Y. P., Goldman, I. F., Udhayakumar V, Collins, W.E., Lal, A.A. & Alpers, M. P. 1993. Identification of *Plasmodium vivax*-like human malaria parasite. *Lancet*, 341, 780-783.

Queller, D.C., Strassmann, J.E. & Hughes C.R. 1993. Microsatellites and kinship. *Trends Ecol Evol*, 8, 285-288.

Raghavendra, K., Barik, T. K., Reddy, B. P., Sharma, P. & Dash, A. P. 2011. Malaria vector control: from past to future. *Parasitol Res*, 108, 757-779.

Rajahram, G. S., Barber, B. E., William, T., Menon, J., Anstey, N. M. & Yeo, T. W. 2012. Deaths due to *Plasmodium knowlesi* malaria in Sabah, Malaysia:

association with reporting as *Plasmodium malariae* and delayed parenteral artesunate. *Malar J*, 11, 284.

Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. & Cowman, A. F. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, 403, 906-909.

Rehman AM1, Mann AG, Schwabe C, Reddy MR, Roncon Gomes I, Slotman MA, Yellott L, Matias A, Caccone A, Nseng Nchama G & Kleinschmidt I. 2013 Five years of malaria control in the continental region, Equatorial Guinea. *Malar J*, 7;12:154.

Reyburn, H., Mbakilwa, H., Mwangi, R., Mwerinde, O., Olomi, R., Drakeley, C. & Whitty, C. J. 2007. Rapid diagnostic tests compared with malaria microscopy for guiding outpatient treatment of febrile illness in Tanzania: randomised trial. *BMJ*, 334, 403.

Rich, S. M., Licht, M. C., Hudson, R. R. & Ayala, F. J. 1998. Malaria's eve: evidence of a recent population bottleneck throughout the world populations of *Plasmodium falciparum*. *Proc Natl Acad Sci USA*, 95, 4425-4430.

Richie, T. L. 1988. Interactions between malaria parasites infecting the same vertebrate host. *Parasitol*, 96, 607-639.

Rogers, D. W., Baldini, F., Battaglia, F., Panico, M., Dell, A., Morris, H. R. & Catteruccia, F., 2009. Transglutaminase-mediated semen coagulation controls sperm storage in the malaria mosquito. *PLoS Biol*, 7, 12.

Rogerson, S.J. & Carter, R. 2008. Severe *Vivax* Malaria: Newly Recognised or Rediscovered? *PLoS Med*, 5, e136.

Roper, C., Pearce, R., Bredenkamp, B., Gumede, J., Drakeley, C., Mosha, F., Chandramohan, D. & Sharp, B. 2003. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet*, 361, 1174-1181.

Rosenberg, R., Wirtz, R. A., Lanar, D. E., Sattabongkot, J., Hall, T., Waters, A. P. & Prasittisuk, C. 1989. Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax*. *Science*, 245, 973-976.

Rowe, J. A., Opi, D. H. & Williams, T. N. 2009. Blood groups and malaria: fresh insights into pathogenesis and identification of targets for intervention. *Curr Opin Hematol*, 16, 480-487.

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., Owour, B., Luckhart, S., Wirtz, R. A., Barnwell, J. W. & Rosenberg, R. 2006. Evidence for transmission of *Plasmodium vivax* among a duffy antigen negative population in Western Kenya. *Am J Trop Med Hyg*, 75, 575-581.

Schnitger, A.K., Kafatos, F.C. & Osta, M.A. 2007. The melanisation reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. *J Biol Chem*, 282, 21884-21888.

Schwartz, L., Brown, G. V., Genton, B. & Moorthy, V. S. 2012. A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malar J*, 11, 11.

Scott, J.A., Brogdon, W.G. & Collins, F.H. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg*, 49, 520-529.

Shah, N. K., Dhillon, G. P. S., Dash, A. P., Arora, U., Meshnick, S. R. & Valecha, N. 2011. Antimalarial drug resistance of *Plasmodium falciparum* in India: changes over time and space. *Lancet*, 11, 57-64.

Silveira, H., Gabriel, A., Ramos, S., Palma, J., Felix, R., Custódio, A. & Collins, L. V. 2012. CpG-containing oligodeoxynucleotides increases resistance of *Anopheles* mosquitoes to *Plasmodium* infection. *Insect Biochem Mol Biol*, 42, 758-765.

Singh, M., Brown, G. & Rogerson, S. J. 2013. Ownership and use of insecticide-treated nets during pregnancy in sub-Saharan Africa: a review. *Malar J*, 12, 268.

Sinka, M. E. 2013. Global Distribution of the Dominant Vector Species of Malaria. In: S. Manguin, ed. 2013. *Anopheles* mosquitoes – New insights into malaria. Croatia: *InTech*. Ch.4.

Smith, T., Genton, B., Baea, K., Gibson, N., Narara, A. & Alpers, M. P. 2001. Prospective risk of morbidity in relation to malaria infection in an area of high endemicity of multiple species of *Plasmodium*. *Am J Trop Med Hyg*, 64, 262-267.

Smythe, J. A., Coppel, R. L., Day, K. P., Martin, R. K., Oduola, A. M., Kemp, D. J. & Anders, R. F. 1991. Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2. *Proc Natl Acad Sci USA*, 88, 1751-1755.

Snounou, G. & White, N. J. 2004. The co-existence of *Plasmodium*: sidelights from *falciparum* and *vivax* malaria in Thailand. *Trends in Parasitol*, 20, 333-339.

Snounou, G., Pinheiro, L., Antunes, A. M., Ferreira, C. & do Rosario, V. E.1998. Nonimmune patients in the Democratic Republic of Sao Tome e Principe reveal a high level of transmission of *P. ovale* and *P. vivax* despite low frequency in immune patients. *Acta Trop*, 70, 197-203.

Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinheiro, L., do Rosario, V. E., Thaithong, S. & Brown, K. N. 2003. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol*, 61, 315-320.

Stephens, J. W. 1922. A new malaria parasitic of man. *Ann Trop Med Parasitol*, 16, 383-388.

Sutherland, C. J., Tanomsing, N., Nolder, D., Oguike, M., Jennison, C., Pukrittayakamee, S., Dolecek, C., Hien, T. T., do Rosário, V. E., Arez, A. P., Pinto, J., Michon, P., Escalante, A. A., Nosten, F., Burke, M., Lee, R., Blaze, M., Otto, T. D., Barnwell, J. W., Pain, A., Williams, J., White, N. J., Day, N. P., Snounou, G., Lockhart, P. J., Chiodini, P. L., Imwong, M. & Polley, S. D. 2010. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. *J Infect Dis*, 201, 1544-1550.

Talisuna, A. O., Nalunkuma-Kazibwe, A., Langi, P., Mutabingwa, T. K., Watkins, W. W., Van Marck, E., Egwang, T. G. & D'Alessandro, U. 2004. Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene

predict sulphadoxinepyrimethamine parasitological failure in Ugandan children with uncomplicated *falciparum* malaria. *Infect Genet Evol*, 4, 321-327.

Tchuinkam, T., Simard, F., Lele-Defo, E., Tene-Fossog, B., Tateng-Ngouateu, A., Antonio-Nkondjio, C., Mpoame, M., Toto,J. C., Njiné, T., Fontenille, D. & Awono-Ambéné, H. P. 2010. Bionomics of *Anopheline* species and malaria transmission dynamics along an altitudinal transect in Western Cameroon. *BMC Infect Dis*, 10, 119.

Temu, E. A., Minjas, J. N., Tuno, N., Kawada, H. & Takagi, M. 2007. Identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group and their role in *Plasmodium falciparum* transmission in Bagamoyo coastal Tanzania. *Acta Trop*, 102, 119-125.

Theopold, U., Schmidt, O., Söderhäll, K. & Dushay, M. S. 2004. Coagulation in arthropods: defence, wound closure and healing. *Trends Immunol*, 25, 289-294.

Thompson, P. E. & Werbel, L. M. 1972. Antimalarial agents: chemistry and pharmacology. *Medicinal chemistry*. Vol. 12. New York: Academic Press.

Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana, M., Lampah, D. A. & Price, R. N. 2008. Multidrug-Resistant *Plasmodium vivax* associated with severe and fatal malaria: A prospective study in Papua, Indonesia. *PLoS Med*, 5, e128.

Tournamille, C., Colin, Y., Cartron, J. P. & Le Van Kim, C. 1995. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet*, 10, 224-228.

Walker, K. & Lynch, M. 2007. Contributions of Anopheles larval control to malaria suppression in tropical Africa: review on achievements and potential. *Med Vet Entomol*, 21, 2-21.

Wang, R., Liang, Z., Hall, M. & Söderhäll. K. 2001. A transglutaminase involved in the coagulation system of the freshwater crayfish, Pacifastacus leniusculus. Tissue localisation and cDNA cloning. *Fish Shellfish Immunol*, 11, 623-637.

Weatherall, D. J. & Clegg, J. B. 2002. Genetic variability in response to infection: malaria and after. *Genes Immun*, 3, 331-337.

Wellems, T. E. & Plowe, C. V. 2001. Chloroquine-resistant malaria. *J Infect Dis,* 184, 770-776.

Whitten, M. M. A., Shiao, S. W. & Levashina, E.A, 2006. Mosquito Midguts and malaria: cell biology, compartimentalization and immunology. *Parasite Immunol*, 28, 121-130.

WHO 2012. World Malaria Report 2012. Geneve, Switzarland: World Health Organization.

WHO 2013. World Malaria Report 2013. Geneve, Switzarland: World Health Organization.

Wilkes, T. J., Matola, Y. G. & Charlwood, J. D. 1996. *Anopheles rivulorum*, a vector of human malaria in Africa. *Med Vet Entomol*, 10, 108-110.

Williams, T. N. 2006. Red blood cell defects and malaria. *Mol Biochem Parasitol*, 149, 121-127.

Williams, T. N., Maitland, K., Bennett, S., Ganczakowski, M., Peto, T. E. A., Newbold, C. I., Bowden, D. K., Weatherall, D. J. & Clegg, J. B. 1996. High incidence of malaria in α-thalassaemic children. *Nature*, 383, 522-525.

Wongsrichanalai, C., Barcus, M. J., Muth, S., Sutamihardja, A. & Wernsdorfer, W. H. 2007. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *Am J Trop Med Hyg*, 77, 119-127.

Yassine, H. & Osta, M. A. 2010. *Anopheles gambiae* innate immunity. *Cell Microbiol*, 12, 1-9.

Yeh, M-S., Huang, C-J., Leu, J-H., Lee, Y. C. & Tsai, I-H. 1999. Molecular cloning and characterization of a hemolymph clottable protein from tiger shrimp (Penaeus monodon). *Eur J Biochem*, 266, 624-633.

Zimmerman, P. A., Mehlotra, R. K., Kasehagen, L. J. & Kazura, J. W. 2004. Why do we need to know more about mixed *Plasmodium* species infections in humans? *Trends in Parasitol*, 20, 440-447.

Aims of this thesis

Malaria continues to be one of the main global public health problems, affecting mainly the poorest areas of the world. Malaria system is very complex with many intervening and interacting factors, namely three biological entities, environmental and socio-economic conditions. A comprehensive analysis which may integrate all of these factors is hard, if not totally impossible, and the majority of the studies so far have been addressing only small pieces of the puzzle. However, it is our belief that a strong effort should be made to approach the problem in a comprehensive way. Transmission of *Plasmodium* parasites by their anopheline vectors is a crucial factor determining the epidemiology of malaria in endemic areas. A better knowledge of the dynamics of transmission would provide further insights in planning and assessing the impact of current and future control strategies.

Besides, another key feature in human malaria epidemiology is parasite diversity, in terms of species or within species populations (different genotypes). In endemic areas, the simultaneous presence of more than one *Plasmodium* species or population both in human and vector hosts is common and these different parasite populations can interact between them, affecting transmission or being a source of selection on pathogen traits such as drug resistance.

The present work aimed to analyse this complex system, studying the population diversity of *Plasmodium* sp andcomparing parasite populations (*Plasmodium* species and *P. falciparum* and *P. vivax* genotypes) circulating in the mosquito vector and human hosts in the same area at the household level.

In addition, the mosquito vector by the activation of a number of defence mechanisms, control malaria infection by limiting the number of parasites during the sporogonic cycle. In fact, it also may act as a source of selection on parasite traits or on the other way round, parasite diversity may have some effect on the establishment of the immune response.

Therefore, in order to get some insights on the above questions, the following Specific Objectives were stated:

Specific objectives

1 – To characterise circulating populations of parasites in both hosts - human and mosquitoes, both regarding *Plasmodium* species and *P. falciparum* and *P. vivax* populations (merging data from surface antigen coding-genes, microsatellite analysis and drug resistance markers);

2 – To analyse selective pressures acting on antimalarial resistance associated *P. falciparum* genes (inferring on origin and spread of mutations).

3 – To analyse the genetic diversity of two genes of *Anopheles gambiae* s.s. - *transglutaminase 1* and *2* genes, inferring their putative effect on the presence / absence of infection.

4 – Finally, to contribute to the description of malaria epidemiology in mainland Equatorial Guinea, particularly regarding *P. falciparum* resistance to antimalarials.

Chapter 2 – Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both humans and mosquito host in continental Equatorial Guinea

This chapter was published as a research paper:

Mendes C., Salgueiro P., Gonzalez V., Berzosa P., Benito A., do Rosário V.E., de Sousa B., Cano J., Arez A.P. (2013) Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea. *Malaria Journal*; 12:114

.

Mendes et al. Malaria Journal 2013, 12:114
http://www.malariajournal.com/content/12/1/114

MALARIA JOURNAL

RESEARCH Open Access

Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea

Cristina Mendes[1], Patrícia Salgueiro[1], Vicenta Gonzalez[2], Pedro Berzosa[2], Agustin Benito[2], Virgílio E do Rosário[1], Bruno de Sousa[3], Jorge Cano[2] and Ana Paula Arez[1*]

Abstract

Background: In *Plasmodium*, the high level of genetic diversity and the interactions established by co-infecting parasite populations within the same host may be a source of selection on pathogen virulence and drug resistance. As different patterns have already been described in humans and mosquitoes, parasite diversity and population structure should be studied in both hosts to properly assess their effects on infection and transmission dynamics. This study aimed to characterize the circulating populations of *Plasmodium* spp and *Plasmodium falciparum* from a combined set of human blood and mosquito samples gathered in mainland Equatorial Guinea. Further, the origin and evolution of anti-malarial resistance in this area, where malaria remains a major public health problem were traced.

Methods: *Plasmodium* species infecting humans and mosquitoes were identified by nested-PCR of chelex-extracted DNA from dried blood spot samples and mosquitoes. Analysis of *Pfmsp2* gene, anti-malarial-resistance associated genes, *Pfdhps*, *Pfdhfr*, *Pfcrt* and *Pfmdr1*, neutral microsatellites (STR) *loci* and *Pfdhfr* and *Pfdhps* flanking STR was undertaken to evaluate *P. falciparum* diversity.

Results: Prevalence of infection remains high in mainland Equatorial Guinea. No differences in parasite formula or significant genetic differentiation were seen in the parasite populations in both human and mosquito samples. Point mutations in all genes associated with anti-malarial resistance were highly prevalent. A high prevalence was observed for the Pfdhfr triple mutant in particular, associated with pyrimethamine resistance.
Analysis of *Pfdhps* and *Pfdhfr* flanking STR revealed a decrease in the genetic diversity. This finding along with multiple independent introductions of *Pfdhps* mutant haplotypes suggest a soft selective sweep and an increased differentiation at *Pfdhfr* flanking microsatellites hints a model of positive directional selection for this gene.

Conclusions: Chloroquine is no longer recommended for malaria treatment in Equatorial Guinea but sulphadoxine-pyrimethamine (SP) remains in use in combination with artesunate and is the only drug recommended in preventive chemotherapy in pregnancy. The high prevalence of point mutations in *Pfdhfr* and *Pfdhps* points to the danger of an eventual reduction in the efficacy of SP combined therapy in *P. falciparum* populations in Equatorial Guinea and to the essential continuous monitoring of these two genes.

Keywords: Malaria, Equatorial Guinea, Genetic diversity, Drug resistance, *pfcrt*, *pfdhps*, *pfdhfr*, *pfmdr1*, Microsatellites, *Plasmodium falciparum*

* Correspondence: aparez@ihmt.unl.pt
[1]Centro de Malária e outras Doenças Tropicais, Unidade de Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal
Full list of author information is available at the end of the article

BioMed Central

© 2013 Mendes et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background

Malaria continues to be one of the main public health problems in the world, affecting 106 countries, with approximately 216 million cases resulting in 650,000 yearly deaths [1]. This parasitic disease involves three living entities with complex interactions among them and transmission of *Plasmodium* parasites by their anopheline vectors is a crucial factor determining the epidemiology of malaria in endemic areas.

The level of genetic diversity of natural populations of *Plasmodium* is well demonstrated and both inter- and intra-specific mixed infections in the same host are common, especially in highly endemic areas [2]. The ecological interactions that these different and co-infecting parasite populations establish among them may be a source of selection on pathogen traits such as virulence and drug resistance.

Parasite genetic diversity and population structure in both humans and mosquitoes should be assessed in order to better determine the influence of different parasite populations on infection and transmission dynamics. In fact, both different associations of *Plasmodium* species as well as marked differences in the multiplicity of infection and allele diversity of *Plasmodium falciparum* populations were previously reported [3]. Furthermore, a recent analysis of both human peripheral blood samples and mosquitoes from the same location has revealed a completely unexpected picture related to the presence of *Plasmodium vivax* in an area where it had not yet been reported [4]. Differences have also been found in drug-resistant associated genes. In Gabon, Mharakurwa *et al* [5] reported that parasites in humans presented high levels of pyrimethamine (PYR)-resistant mutants, whereas parasites in *Anopheles* mosquitoes showed high levels of cycloguanil-resistant mutants.

For a period of time, the genetic diversity of *P. falciparum* populations has mainly been investigated through the analysis of mutation in polymorphic surface antigen coding genes [6,7]. However, this approach poses some limitations as it is impossible to know whether observed patterns reflect population history or natural selection [8]. Microsatellite sequences (STR), spread throughout the genome, are currently the neutral markers most commonly used to differentiate *P. falciparum* populations as these markers (short repeated nucleotide sequences) often present high levels of inter- and intra-specific polymorphism, particularly when the number of repetition is 10 or higher.

In Equatorial Guinea, malaria remains the major endemic disease and the leading cause of child mortality and morbidity. In recent years, the prevalence of infection has been reduced significantly on the Insular Region due to an effective vector control [1,9] whilst the prevalence of infection remains above 50% in children under five years old in mainland region [10]. Along with the high prevalence of infection, the dissemination of *P. falciparum* drug resistance still remains the main constraint to control malaria transmission in most endemic areas. Anti-malarial resistance has largely been studied through the analysis of mutations on several target genes associated with resistance to specific drugs, e g, *Pfcrt* [11] and *Pfmdr1* [12] linked to chloroquine (CQ) resistance; and *Pfdhfr* [13] and *Pfdhps* genes [14] associated with resistance to pyrimethamine (PYR) and sulphadoxine (SFX), respectively.

Increasing failure rates (40-50%) for CQ and around 25% resistance to sulphadoxine/pyrimethamine (SP) in under-five children was reported in 2003 in Malabo, the capital city of Equatorial Guinea located in the island of Bioko [15]. Nevertheless, CQ continued to be used in mainland region as the first-line treatment for uncomplicated malaria until 2009, and had been replaced by artesunate + sulphadoxine/pyrimethamine (AS+SP) combination on the island of Bioko in 2004 [16]. In 2009, artemisinin combination therapy (ACT) of artesunate/amodiaquine (AS/AQ) was adopted as first-line therapy based on the high levels of resistance to SP in neighbouring countries. More recently, a study conducted in Bata, the largest city in the mainland region, and Malabo revealed that AS/SP and AQ/SP combinations were both highly effective for the treatment of uncomplicated *P. falciparum* malaria [16]. SP is still prescribed alone for intermittent preventive therapy in pregnant women [17].

This study aimed to characterize the circulating populations of *Plasmodium* spp and *P. falciparum* from a combined set of human blood and mosquito samples collected in both coastal and inland villages from mainland Equatorial Guinea. *Plasmodium falciparum* diversity was analysed through the study of an antigen coding gene (*Pfmsp2*) as well as a set of neutral STR *loci* and four anti-malarial resistant associated genes (*Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps*). Finally, to trace the origin of anti-malarial resistance and its progression in this area, the presence of signatures of drug resistant selection in *P. falciparum* populations were investigated. The impact of these findings on control policies, especially the avoidance of dissemination of *P. falciparum* drug-resistant parasites in Equatorial Guinea, is discussed.

Methods

Sample collection

Peripheral blood samples from 97 inhabitants (zero to 78 years-old) were collected in 2005 in mainland Equatorial Guinea during the dry (February and August) and rainy (May) seasons from two villages, Miyobo (34 and 43 individuals in the dry and rainy seasons, respectively; 44 different individuals in total) and Ngonamanga (40 and 26 individuals in the dry and rainy seasons, respectively; 53 different individuals in total). Blood sampling has been performed in four consecutive days per individual,

in order to better assess variations in the *P. falciparum* population's composition. Further, 819 mosquito specimens were also collected during the same period and locations. Miyobo is located in a forested area on the riverbank of the Wele River, which crosses the mainland region from east to west. Ngonamanga is a coastal village surrounded by forest-savannah, 60 km north of Bata. In both, malaria is classified as hyperendemic. Both study areas and sample collection procedures have been described elsewhere [4].

Villagers were informed of the nature and aims of the study and voluntary participation of five households randomly selected by location was requested after approval of local authorities. Blood samples were collected after informed consent was received from all donors (parents or guardians responded on behalf of children). Mosquito collection was done after the approval of local authorities, the owner and occupants of the house. Written consent was obtained from the legal guardians of the recruited children and non-documented, oral consent was only requested from adults, due to the community-wide mistrust of signing official forms. The study was approved by the Ethical Committee of Equatorial Guinea's Ministry of Health and Social Welfare, the National Malaria Control Programme, and local health authorities from the villages, which accepted the constraint and found no bio-ethical impediments to the study. Ethical clearance was also given by the Ethical Committees of the Instituto de Higiene e Medicina Tropical (Lisboa, Portugal) and the Instituto de Salud Carlos III (Madrid, Spain), according to EU regulations.

DNA extraction and molecular assays

Individual mosquitoes, dried on silica gel, and blood spot samples were stored at room temperature until DNA preparation. DNA was extracted using chelex according to Plowe *et al* [18] from blood spots and to Arez *et al* [19] from mosquitoes.

Detection of malaria infection and identification of *Plasmodium* species was carried out by nested-PCR amplification of the ssrRNA genes [20]. *Plasmodium falciparum* positive samples were further genotyped for:

a) *Pfmsp2* gene by a nested-PCR as in Snounou *et al* [21];
b) Drug resistant associated genes by a nested PCR-RFLP analysis of the presence/absence of mutations at codons 75 and 76 of the *Pfcrt* gene, codons 86 and 1246 of the *Pfmdr1* gene, codons 51, 59, 108 and 164 of the *Pfdhfr* gene and codons 436, 437, 540 and 581 of the *Pfdhps* gene [22],
c) Nine neutral microsatellite *loci* (STR) distributed throughout the genome of *P. falciparum*: TAA42, TAA81 (chromosome 5), TA1, TAA87, TAA109 (chromosome 6), ARA2 (chromosome 11), TA102, PfPK2 and Pfg377 (chromosome 12). Primer sequences and PCR conditions are described in Anderson *et al* [23] and Conway *et al* [24];
d) STRs flanking *Pfdhfr and Pfdhps* genes located 0.3 kb, 4.4 kb and 5.3 kb upstream of codon 108 of *Pfdhfr* (chromosome 4) and 0.8 kb, 4.3 kb and 7.7 kb downstream from codon 437 of *Pfdhps* (chromosome 8). Primer sequences and PCR conditions are described in Roper *et al* [25], Ndiaye *et al* [26] and Salgueiro *et al* [27]. Southeast Asian *P. falciparum* K1 laboratory strain was used as reference (at STRs flanking the *Pfdhps* gene, the allelic composition of the K1 strain matches that of the East African *Pfdhps* double mutant A437G/K540E haplotype lineage SGE 1 [28]).

Amplified fragments were run in an automatic sequencer (ABI 3730, Applied Biosystem) at Yale University, DNA Analysis Facility on Science Hill. The software GeneMarker (SoftGenetics) was used to measure allele sizes. Samples that failed the amplification in any of the *loci* or presented multiple STR peaks were excluded for the haplotype definition [25]. A new haplotype was considered when there was one or more allelic changes across all *loci* considered. For the remaining analyses, in cases where multiple peaks were present, only the value of the highest peak per locus was scored [8].

Statistical analysis

Pearson χ^2 test was used to compare prevalence of infection between collection sites, seasons and hosts. Whenever Pearson χ^2 test conditions were not satisfied, Fisher's exact test was used (SPSS v.12 statistical software). Pearson's χ^2 test was also used to assess possible associations between *Plasmodium* species [29].

Prevalence of *Pfmsp2* alleles and the minimum number of concurrent genotypes in the same isolate (multiplicity of infection (MOI): the largest number of alleles found in each sample) were calculated for all comparison groups; mosquitoes *versus* blood samples, Miyobo *versus* Ngonamanga and rainy season *versus* dry season.

STR data was analysed with FSTAT v. 2.9.3.2 [30] to obtain measures of genetic diversity [allelic richness Rs: a measure of the number of alleles independent of sample size, hence allowing to compare this quantity between different sample sizes; and expected heterozygosity He per locus and sample: this use an unbiased estimator Hs, which is calculated from individual allele frequencies and range from zero (no heterozygosity) to nearly 1.0 (for a system with a large number of equally frequent alleles)] and genetic differentiation using the Fst estimator. Linkage disequilibrium (LD) tests were performed with GENEPOP v.3.4 [31].

After the assessment of PYR- and SFX-associated wild type (or sensitive) and mutant alleles, comparisons were made between populations classified as "wild type", "single mutant" (*Pfdhfr*: 51 or 59 or 108 or 164; *Pfdhps:* 436 or 437 or 540 or 581), "double mutant" (*Pfdhfr:* 51:108 or 59:108 or 51:59 or 51:164; *Pfdhps*: 436:581 or 437:581 or 540:581), "triple mutant" (*Pfdhfr*: 51:59:108 or 59:108:164 or 51:59:164, *Pfdhps:* 436:437:581 or 436:540:581), "quadruple mutant" (*Pfdhfr*: 51:59:108:164, *Pfdhps* 436:437:540:581). However this was not always possible due to the low number of samples in some groups, so that only the whole sample was subdivided and compared according to geographic collection sites.

In multiple tests, Bonferroni correction was applied by dividing 0.05 by the number of tests to minimize type I errors and obtain the actual cut-off for significance [32].

Results

Detection and identification of *Plasmodium* species

A total of 427 blood samples from 97 individuals were collected in both villages and seasons (44 individuals from Miyobo and 53 from Ngonamanga). A total of 819 mosquitoes were collected (509 from Miyobo and 310 from Ngonamanga), 536 belonging to *Anopheles gambiae* complex, 259 belonging to *Anopheles nili* complex (presumably *Anopheles carnevalei*), three to *Anopheles funestus* complex and 21 *Anopheles moucheti moucheti*.

In order to determine prevalence of infection, an individual was defined as infected if he/she had at least one positive sample among the multiple samples collected; therefore, only one sample was considered per individual and all calculations were performed having the number of individuals as denominator. Overall, prevalence of *Plasmodium* spp infection in humans was 93% in Miyobo and 81% in Ngonamanga, and was higher in the dry season (69%) than in the rainy (67%). In mosquitoes, the prevalence of infection was slightly higher in Ngonamanga (20%) than Miyobo (19%) and in the rainy season (22%) than in the dry season (16%). Although the four *Plasmodium* species were detected in both hosts, *P. falciparum* was the predominant species occurring in 90% of the isolates (both humans and mosquitoes) either in single or mixed infection (see Additional files 1 and 2). In humans, *Plasmodium malariae* was the second most prevalent species, occurring in 13% of individuals, followed by *P. vivax* (10%) and finally *Plasmodium ovale* (8%) (see Additional file 1). In mosquitoes, *P. vivax* was the second most prevalent species (9%), followed by *P. malariae* (4%) and *P. ovale* (2%) (see Additional file 2). A significantly higher number than expected of mixed infections with *P. falciparum* and *P. malariae* in both hosts (blood samples: χ^2=8.973, p=0.003; mosquitoes: χ^2=15.745, p<0.001) was found. No association was found for the pair *P. falciparum* and *P. viv*ax.

Plasmodium falciparum genetic diversity

Pfmsp2

Plasmodium falciparum was detected in 302 out of the 427 samples collected and successful genotyping of *Pfmsp2* gene was achieved in 73% (221/302) *P. falciparum*-positive blood samples and none in the 275 *P. falciparum*-positive mosquitoes. The unsuccessful amplification of *Pfmsp2* in mosquitoes was probably due to degradation of parasite DNA in dried mosquitoes stored at room temperature for a long period of time.

No major differences in allelic diversity were detected between seasons or villages, which shared 11 out of 13 alleles; two unique alleles were detected in Ngonamanga in dry season (IC_400 and IC_700) and only one was observed in the rainy season (FC27_250) in both villages. The mean MOI was slightly higher in Miyobo than in Ngonamanga; 1.98 *versus* 1.83, respectively, and varied between 1.46 (Ngonamanga, rainy season) and 2.19 (Ngonamanga, dry season). When values are compared between villages without season distinction, mean MOI was slightly higher in Miyobo than in Ngonamanga (1.98 *versus* 1.83, respectively) and it was higher in the dry season in Ngonamanga (dry *versus* rainy: 2.19 versus 1.46), while the opposite occurred in Miyobo (dry *versus* rainy: 1.88 *versus* 2.07).

Neutral STRs loci

Ninety-nine per cent (299/302) *P. falciparum*-positive blood samples and 83% (228/275) *P. falciparum*-positive mosquitoes were successfully genotyped. The number of observed alleles (Na), allelic richness (Rs) and genetic diversity (uH) are shown in Table 1. All nine STR analysed were polymorphic and the number of alleles varied between seven in Pfg377 and 17 in TA109 in human samples, and six in TA42 and 18 in TA109 in mosquitoes. The majority of samples presented multiple *P. falciparum* genotypes but in general, the most common alleles are shared between parasite populations present in humans and mosquitoes. Genetic diversity (uH) also presented similar values; 0.75 *versus* 0.77, in humans and mosquitoes, respectively, as well as the number of alleles for each *locus*; 12 *versus* 11, in humans and mosquitoes respectively (see Table 1).

MOI varied between 1.62 (Miyobo, dry season, mosquitoes) and 2.25 (Ngonamanga, dry season, humans) and tended to be higher in humans than in mosquitoes (2.09 *versus* 1.80, respectively). When values are compared between villages without season distinction, mean MOI in humans was slightly higher in Ngonamanga (2.07) than in Miyobo (2.11), and conversely in mosquitoes;

Table 1 Neutral microsatellite diversity of *Plasmodium falciparum* populations from Ngonamanga and Miyobo in humans and mosquitoes

		n		TA1	TA102	ARA2	TA87	Pfk2	TA81	TA42	Pfg377	TA109	Mean
Humans	**TBs**	299	**Na**	13	12	9	15	14	11	14	7	17	12
			Rs	12	11	8	12	13	10	10	5	13	10
			uH	0.852	0.832	0.787	0.838	0.877	0.829	0.344	0.612	0.799	0.75
	BsM	195	**Na**	11	11	9	14	12	11	7	7	15	11
			Rs	10	9	8	9	10	9	5	5	9	8
			uH	0.846	0.848	0.771	0.829	0.857	0.792	0.293	0.642	0.811	0.74
	BsN	104	**Na**	11	8	8	9	11	10	11	5	9	9
			Rs	10	7	8	8	11	10	7	5	7	8
			uH	0.859	0.771	0.809	0.844	0.877	0.868	0.424	0.531	0.747	0.75
	BsW	141	**Na**	10	11	7	13	12	11	8	5	9	10
			Rs	9	10	7	10	11	10	6	5	8	8
			uH	0.819	0.854	0.797	0.840	0.874	0.851	0.307	0.634	0.805	0.753
	BsD	158	**Na**	11	10	9	11	12	10	11	7	13	10
			Rs	10	8	8	9	11	9	8	5	9	9
			uH	0.866	0.817	0.766	0.841	0.871	0.798	0.375	0.592	0.788	0.746
Mosquitoes	**TMq**	228	**Na**	13	12	8	11	12	10	6	8	18	11
			Rs	12	12	8	11	12	10	6	7	16	10
			uH	0.88	0.874	0.810	0.849	0.833	0.779	0.365	0.712	0.869	0.77
	MqM	130	**Na**	12	11	7	9	12	9	6	6	15	10
			Rs	10	11	7	8	10	8	6	5	13	9
			uH	0.851	0.870	0.780	0.845	0.845	0.753	0.462	0.713	0.848	0.77
	MqN	98	**Na**	9	7	7	8	8	8	3	6	14	8
			Rs	9	7	7	8	8	8	3	6	11	7
			uH	0.862	0.815	0.760	0.814	0.816	0.747	0.191	0.718	0.837	0.73
	MqW	86	**Na**	11	11	5	9	12	9	6	7	8	9
			Rs	10	11	5	9	11	9	6	6	8	8
			uH	0.882	0.904	0.741	0.823	0.846	0.774	0.428	0.717	0.808	0.769
	MqD	142	**Na**	10	7	8	9	10	8	4	5	17	9
			Rs	10	7	8	9	10	8	4	5	14	8
			uH	0.873	0.796	0.834	0.826	0.825	0.788	0.300	0.713	0.873	0.758

n*:** sample size; ***TBs*:** total of blood samples; ***BsM*:** Blood samples from Miyobo; ***BsN*:** Blood samples from Ngonamanga; ***BsW*:** Blood samples wet season; ***BsD*:** Blood samples dry season; ***TMq*:** total mosquitoes; ***MqM*:** Mosquitoes from Miyobo; ***MqN*:** Mosquitoes from Ngonamanga; ***MqW*:** Mosquitoes wet season; ***MqD*:** Mosquitoes dry season; ***Na: number of observed alleles; ***Rs***: allelic richness; ***uH*:** unbiased estimation of genetic diversity.

1.69 and 1.91, Ngonamanga and Miyobo respectively. No significant genetic differentiation was observed among all study groups.

Drug resistant associated genes

SNPs: Pfcrt, Pfmdr1, Pfdhfr and Pfdhps

No major differences in the prevalence of mutant alleles were found among villages or seasons for any of the genes. Regarding *Pfcrt* and *Pfmdr1* genes associated with CQ resistance, the prevalence of the *Pfcrt* mutant alleles (N75E and K76T), present in single or mixed infection, was 56% and 72% in humans and 64% and 54% in mosquitoes; and a much higher prevalence of mutation in codon N86Y (84% and 61%, in humans and mosquitoes, respectively) than in D1246Y (1% in both hosts) was found in *Pfmdr1* gene (see Additional file 3). Regarding *Pfdhfr* gene, mutations N51I, C59R and S108N, associated with PYR resistance, presented prevalence, when in single or mixed infection, of 73%, 85%, 93% in humans and 81%, 81%, 95% in mosquitoes, respectively. The codon I164L was found in very low frequency (15% in humans and 0 in mosquitoes) (see Figure 1 and Additional file 3). While in Miyobo the double mutation (C59R/S108N) was the most prevalent in Ngonamanga

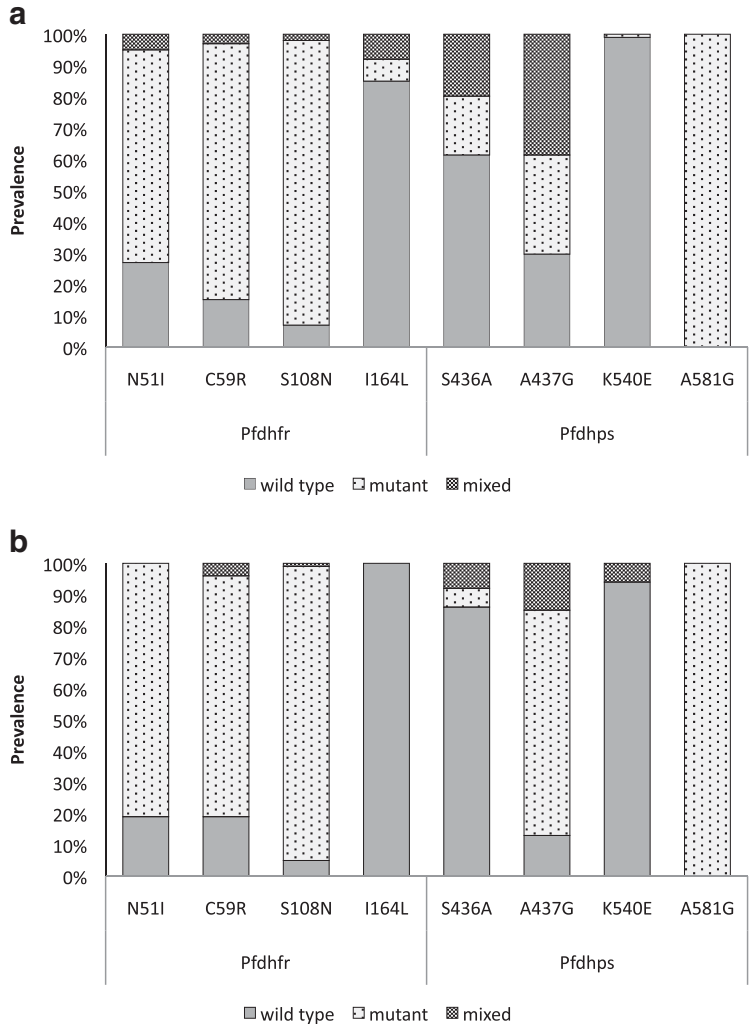

Figure 1 Total prevalence of mutations in the eight codons of *Pfdhfr* and *Pfdhps* genes. Legend: Prevalence of mutations in the *Pfdhfr* (N51I, C59R, S108N, I164L) and *Pfdhps* (S436A, A437G, K540, 581G), in single (wild type or mutant) and mixed infections in both seasons and localities in humans (**a**) and mosquitoes (**b**).

most samples contained the triple mutation (N51I/C59R/S108N).

Mutations A437G and A581G in *Pfdhps* gene associated with SFX resistance were detected at very high prevalence, the latter reaching 100%, whereas a very low prevalence of codon K540E was found (1% in humans and 6% in mosquitoes only in mixed infections) (see Figure 1 and Additional file 3). Mutation S436A occurred in 38% in humans and 14% in mosquitoes. When comparing the two villages, no major differences were found in mosquitoes, and the prevalence of mutant alleles were: 12% in Ngonamanga and 20% in Miyobo, unlike in humans where the prevalence of this mutation was higher in Ngonamanga (51%) than in Miyobo (31%).

Most samples presented the double mutation (A437G/A581G), but many samples (approximately 38%) containing the triple mutation (A436/G437/G581) were identified in Ngonamanga.

Despite the high prevalence of resistance-associated mutations in *Pfdhfr* and *Pfdhps*, no parasites containing the quintuple mutation (N51I/C59R/S108N/A437G/K540E) associated with the clinical failure of SP combination were found.

STR loci flanking Pfdhfr and Pfdhps genes

The analysis using the STR flanking *Pfdhfr* and *Pfdhps* genes was only conducted in human isolates, since the amplification rate of these *loci* was very low in mosquito samples.

Genetic diversity and linkage disequilibrium

The effect of SP selection on the *P. falciparum* population of Equatorial Guinea was evaluated by examining and comparing the levels of genetic diversity, LD and genetic differentiation between the *Pfdhfr* and *Pfdhps* flanking and neutral STR *loci*. Overall, genetic diversity estimated at neutral *loci* (*He* = 0.75; *Rs* = 14; *N* =244) was higher than at *loci* flanking both *Pfdhfr* (*He* = 0.15; *Rs* = 8; *N* =189) and *Pfdhps* (*He* = 0.80; *Rs* = 12; *N* = 189) genes (see Tables 2 and 3). In parasites holding *Pfdhfr* resistance associated alleles, triple mutants showed lower levels of genetic diversity (0.11) when compared to the single (0.36) and double mutants (0.21) (see Table 2). The mean *He* at three *Pfdhfr loci* was 0.22, which was much lower when compared to the mean *He* at 9 neutral *loci* (0.74) (see Table 2).

The reduction in the genetic diversity is not so marked in *Pfdhps* as in *Pfdhfr*. When double mutants and triple mutants are compared, there is a slight decrease in genetic diversity (*He*=0.81 for double *versus* *He*=0.67 for triple mutants) but values are still similar and high (see Table 3). Statistical tests for LD were conducted for all pairs of flanking STR on each of the mutant groups – single, double and triple mutants (105 possible tests for *Pfdhfr* and 316 for *Pfdhps*). Only two associations showed significant results ($p < 0.05$), after Bonferroni's correction was applied, in the *Pfdhps* double mutants group, involving *loci* 0.8 kb/4.3 kb and 4.3 kb/7.7 kb. No significant pairwise association was found involving the *Pfdhfr* gene.

Pfdhfr and *Pfdhps* haplotype characterization

Only samples with single infections and successful amplification of all *loci* were used for the haplotype characterization. Thus, haplotypes were reconstructed in 57 out of 298 human isolates genotyped for *Pfdhfr* and in 35 out of 296 human isolates genotyped for *Pfdhps*. For the *Pfdhfr* gene, nine distinct haplotypes were found (see Additional file 4). The haplotype H9, an exact match of the *P. falciparum* K1 strain used as a control (double mutation C59R/S108N and allele sizes of 113 bp, 183 bp and 210 bp to the 0.3 kb, 4.4 kb and 5.3 kb *loci*, respectively), was found in 10 samples from Miyobo. Most frequent haplotypes in 53 out of 57 samples (H1, H3, H5, H8) also matched STR sizes in K1 strain.

The majority of samples from Ngonamanga (97%) showed the triple-mutant IRNI (51I:59R:108N:164I – mutated codons appear underlined), while in Miyobo the most prevalent haplotype was the double-mutant NRNI (51N:59R:108N:164I) (43%) followed by the triple-mutant IRNI with 26% (see Additional file 4).

Regarding the *Pfdhps* gene, 25 distinct haplotypes were found (see Additional file 5); 16 in Miyobo and nine in Ngonamanga, only one shared between the two villages. None of the haplotypes found matches with K1 strain (single mutation A437G and allele sizes of 131 bp, 103 bp and 108 bp to the 0.8 kb, 4.3 kb and 7.7 kb *loci*, respectively). The haplotypes found for the *Pfdhps* gene have multiple independent lineages since the majority of the haplotypes were unique. Nevertheless, the most prevalent haplotype in Ngonamanga was the triple-mutant AGKG (436A:437G:540K:581G) with 38%, whilst in Miyobo it was the double-mutant SGKG (436S:437G:540 K:581G) (63%).

Discussion

Malaria still is a major public health concern in Equatorial Guinea, especially in the mainland. In order to contribute to the update of the malaria situation in this area, a combined set of blood and mosquito samples from the same locations were analysed to characterize the genetic diversity of circulating populations of *Plasmodium* spp and especially of *P. falciparum*, in both hosts.

Plasmodium species diversity

This study presents a much higher prevalence of *Plasmodium* infection in mainland Equatorial Guinea (87%) than the one reported for the Insular Region in 2005 (32%) [33]. This difference is likely due to the fact that most malaria control activities have been deployed on the island of Bioko where the capital, Malabo is located. In 2004, the first stage of the project "The Bioko Island Malaria Control Project (BIMCP)" was launched and initial reports stated a significant decrease in the prevalence of infection, achieving an overall malaria prevalence of 18% in 2008 [10,34]. In the present study, although *P. falciparum* infections were the most frequent, *P. vivax* infections were detected for the first time both in humans and mosquitoes, which means that active transmission of this species not previously reported in this area is occurring. The apparent higher presence of *P. vivax* in mosquitoes might be due to its higher visibility in the vector, since in the human host this parasite can form dormant forms in the liver – hypnozoites - and go unnoticed, as discussed in [4].

Regarding mixed infections, *P. falciparum* and *P. malariae* are also associated in mainland Equatorial Guinea, as has been reported in other sub-Saharan countries [3,35-39]. This association was observed both in humans and mosquitoes, which suggests that no differing patterns of *Plasmodium* species association in the two hosts occurs as has formerly been reported in Guinea Bissau [3].

Plasmodium falciparum genetic diversity

Concerning *P. falciparum* genetic diversity, the analysis of both *Pfmsp2* and neutral STR in humans showed similar levels of allelic diversity and MOI in both villages and seasons. No reduction of genotype diversity or MOI was observed with the decline of transmission, as seen

Table 2 Statistics of the 15 STR loci of *Plasmodium falciparum*-positive individuals: mutants to PYR

Microsatellites		Sampled populations - Miyobo										Sampled populations - Ngonamanga	
		Single mutant		Double mutant		Triple mutant		All samples				Triple mutant	
		(*N*=10)		(N=37)		(N=17)		(N=64)				(N=82)	
		R_s	H_e	R_s	H_e	R_s	H_e	R_s	H_e	F_{ST}	*P*	R_s	H_e
***Loci* flanking *dhfr* gene**	*Dhfr* 0.3	3	0.51	3	0.32	2	0.15	3	0.33	0.02	NS	7	0.26
	Dhfr 4.4	1	0.00	2	0.14	2	0.17	2	0.10	−0.04	NS	3	0.10
	Dhfr 5.3	3	0.56	2	0.18	1	0.00	2	0.24	0.10	0.03	3	0.08
	All *loci*	2	0.36	2	0.21	2	0.11	2	0.22	0.03	0.05	4	0.15
Neutral *loci*	TA1	4	0.82	5	0.83	6	0.89	6	0.85	0.04	0.04	12	0.87
	TA102	4	0.64	7	0.88	5	0.84	6	0.79	0.03	NS	8	0.78
	ARA2	4	0.69	5	0.77	6	0.79	5	0.75	−0.03	NS	9	0.81
	TA87	4	0.87	6	0.85	5	0.81	6	0.84	0.01	NS	9	0.84
	PfPK2	5	0.93	6	0.86	5	0.85	6	0.88	−0.03	NS	12	0.87
	TA81	4	0.87	5	0.81	4	0.71	5	0.80	−0.04	NS	10	0.87
	TA42	3	0.60	2	0.11	2	0.28	2	0.32	0.06	NS	8	0.36
	Pfg377	3	0.67	3	0.56	3	0.65	3	0.62	−0.04	NS	7	0.55
	TA109	5	0.86	5	0.80	4	0.79	5	0.82	0.04	0.03	8	0.75
	All *loci*	4	0.77	5	0.72	4	0.73	5	0.74	<0.01	NS	9	0.74

***N*:** number of isolates genotyped, ***He*:** expected heterozygosity; ***Rs*:** allelic richness. **All *loci*:** mean over *loci Rs* and *He* and global *Fst* over *loci* as calculated by FSTAT. ***P*:** *P*-values of permutation tests to assess significance of *Fst* values. **NS:** non-significant (*P*>0.05).

Table 3 Statistics of the 15 STR *loci* of *Plasmodium falciparum*-positive individuals: mutants to SFX

Microsatellites		Sampled populations - Miyobo								Sampled populations - Ngonamanga							
		Single mutant (N=17)		Double mutant (*N*=43)		All samples (*N*=60)				Double mutant (N=63)		Triple mutant (N=22)		All samples (N=85)			
		R_s	H_e	R_s	H_e	R_s	H_e	F_{ST}	*P*	R_s	H_e	R_s	H_e	R_s	H_e	F_{ST}	*P*
***Loci* flanking *dhps* gene**	*Dhps* 0.8	4	0.60	5	0.75	5	0.67	0.02	NS	9	0.81	5	0.64	9	0.73	0.06	0.01
	Dhps 4.3	6	0.83	5	0.77	6	0.80	<0.01	NS	7	0.78	4	0.71	7	0.75	0.13	<0.01
	Dhps 7.7	6	0.79	7	0.84	8	0.82	0.09	<0.01	10	0.85	6	0.67	10	0.76	0.14	<0.01
	All *loci*	5	0.74	6	0.79	6	0.76	0.04	<0.01	9	0.81	5	0.67	9	0.74	0.11	<0.01
Neutral *loci*	TA1	5	0.79	8	0.87	8	0.83	0.04	NS	9	086	8	0.88	9	0.87	<0.01	NS
	TA102	5	0.78	9	0.87	9	0.83	0.04	0.01	6	0.75	7	0.86	7	0.80	<-0.01	NS
	ARA2	6	0.79	6	0.69	7	0.74	0.06	<0.01	7	0.80	6	0.82	7	0.81	−0.01	NS
	TA87	5	0.79	8	0.84	7	0.82	0.05	0.02	8	0.83	7	0.87	8	0.85	0.02	NS
	PfPK2	5	0.83	8	0.80	8	0.81	0.11	0.02	10	0.87	8	0.87	11	0.87	0.02	<0.05
	TA81	5	0.77	6	0.78	7	0.78	−0.02	NS	9	0.88	8	0.83	9	0.85	<0.01	NS
	TA42	1	0.00	4	0.29	3	0.14	0.03	NS	5	0.44	3	0.18	5	0.31	0.05	0.04
	Pfg377	3	0.56	4	0.59	3	0.57	<-0.01	NS	5	0.59	3	0.40	5	0.50	0.01	NS
	TA109	6	0.86	6	0.81	6	0.83	−0.02	NS	5	0.72	5	0.60	6	0.66	0.16	<0.01
	All *loci*	5	0.69	6	0.73	6	0.71	0.03	<0.01	7	0.75	6	0.70	7	0.72	0.03	0.01

***N*:** number of isolates genotyped, ***He*:** expected heterozygosity; ***Rs*:** allelic richness. **All *loci*:** mean over *loci Rs* and *He* and global *Fst* over *loci* as calculated by FSTAT. ***P*:** *P*-values of permutation tests to assess significance of *Fst* values. **NS:** non-significant (*P*>0.05).

in areas of lower endemicity, such as Sudan [40] or in areas with marked differences in malaria endemicity [41]. However, analogous results were obtained by Cano *et al* [42] in a study conducted on the island of Annobon, part of Equatorial Guinea Insular Region.

In mosquitoes, this analysis was only possible with the neutral STR and the results confirmed those obtained in humans, i. e., high levels of genetic diversity and no significant genetic differentiation between geographic locations, despite their different ecological differences or seasons. This is a sign of high malaria endemicity in mainland Equatorial Guinea and the similarity between population genetic structures is concordant with other studies in African highly malaria-endemic countries [8,40]. No significant genetic differentiation was seen between hosts, when comparisons between human blood samples and mosquitoes were made using neutral STR data. The most common alleles are found in both humans and mosquitoes, which may indicate consistency in the parasite populations that are being transmitted. Nevertheless, MOI values were higher in humans than in mosquitoes. As Arez *et al* [3] observed, a higher proportion of single-genotype infections in mosquitoes could point to a limited genetic diversity of the *inocula* and a high genetic diversity in humans resulting from superinfection phenomena.

Anti-malarial resistance evolution

The prevalence of the main point mutations associated with CQ resistance (75E and 76 T of *Pfcrt* gene and 86Y of *Pfmdr1* gene) was nearly 71%. Although the mutation 1246Y in the *Pfmdr1* gene has also been associated with reduced susceptibility to CQ [43], a very low frequency of this mutation was found in Equatorial Guinea (1%).

Nowadays, after the introduction of artemisinin-based combination therapy (ACT), decrease in prevalence of mutations associated with CQ resistance might be expected, due to the absence of drug pressure, as reported in Malawi, China, Kenya and Angola [44-47]. However, a recent study conducted in Equatorial Guinea [48] found higher prevalence of mutation in *Pfcrt* (codon 76) and in *Pfmdr1* (codon 1246) (98% and 96%, respectively), than those found in this study (72% for *Pfcrt* codon 76 and 1% for *Pfmdr1* codon 1246) in isolates collected in 2005, when CQ was still in use in mainland Equatorial Guinea. The increasing of these and other point mutants might be a result of selective pressure by AS-AQ combination, since AQ is a close Mannich base analogue of CQ, promoting the maintenance of CQ-resistant isolates with the mutant *Pfcrt* and *Pfmdr1* genotypes. On the other hand, another possibility is the continuous use of CQ despite national therapeutic guidelines [49].

In Equatorial Guinea, SP has been used as a second-line therapy for many years and lately, though less intensely, as a first-line in combination with artemisinin derivatives and it is used in preventive chemotherapy in pregnancy. Although the failure rate of this combination has not suffered major variations since 1992, and in the late 1990s was still 10% [15], it was expected that the continuous use of this drug would rapidly lead to an increase of resistance levels as had happened in other countries such as Kenya [50] and Tanzania [51].

In fact, a high prevalence of mutation in genes associated with resistance to the SP combination (~70%) was observed in this study. PYR resistance seemed to be well established in mainland Equatorial Guinea and nearly 80% of parasite populations presented the triple mutant N51I/C59R/S108N in the *Pfdhfr* gene, both in humans and mosquitoes, as seen in other nearby countries such as Cameroon [52], Gabon [53] and São Tomé and Principe [27].

Regarding SFX resistance, a high prevalence of the mutation A437G in *Pfdhps* was detected. However the mutation K540E was practically non-existent, which is usual in West Africa [28]. The prevalence of S436A mutation was low, contrary to data from the neighbouring country Gabon, where this was the most frequent *Pfdhps* polymorphism [54]. The mutations S436A and A581G are less studied due to their low prevalence in some African countries, and the lack of knowledge of their role in treatment failure [55]. However, the prevalence of A581G mutation in this study reached 100%. Other recent studies conducted in different African countries showed an increase of the prevalence of A581G, during the last years [53,56].

The quintuple mutant, associated with SP clinical failure [57,58] and resulting from the combination of the *Pfdhfr* triple mutant N51I/C59R/S108N (linked to resistance to PYR) with the *Pfdhps* double mutant A437G/K540E (linked to resistance to SFX), was not detected since no samples containing the latter were found. No major differences in the prevalence of mutation between parasites in humans and mosquitoes occurred.

Analysis on *Pfdhfr* flanking STRs showed that the majority of haplotypes found were associated with triple mutants IRNI, especially in Ngonamanga, while the majority of isolates harboured double mutants NRNI in Miyobo. These two haplotypes have already been reported in Ghana [59] and the triple mutant IRNI was also found in Southeast Asia [60]. The majority of the haplotypes seems to have a single origin. In fact, the haplotypes found were very similar among them, with the majority of them corresponding to the H3 haplotype. This haplotype has arisen from H9 haplotype double mutant through an additional mutation occurring at position 59 of the *Pfdhfr* gene. Both H3 and H9 haplotypes share the same microsatellite profile.

The results of the present study suggest that PYR resistance was firstly established in Ngonamanga, probably due to the fact that Miyobo is more isolated and the introduction of the drug may have occurred later. It is likely that SP combination has been introduced first in Malabo (the capital of the country), and then its utilization was spread all over the country. Ngonamanga, being a coastal area (closer to the capital), may have started to use this drug earlier, and therefore to develop resistance sooner. The process of the addition of a single mutation in *Pfdhfr* alleles to double mutants, originating a high prevalence of triple mutants [61] was still occurring in Miyobo. The most common haplotype 113/183/210 with the triple mutant IRNI, already described in Senegal [26], should be related to the 109/183/210 background, found in Tanzania, South Africa, Southeast Africa [25] and, most recently, in Kenya [50].

It was expected that the extensive use of SP would lead to a rapid increase in resistance levels, leaving signatures of drug selective pressure, such as a reduction in genetic diversity around *Pfdhps* and *Pfdhfr* due to selective sweep; an increased genetic differentiation at the *loci* under selection; and, a significant LD between *loci* flanking *Pfdhps* and *Pfdhfr* genes [62].

Indeed, the reduction in heterozigosity in the *loci* flanking *Pfdhfr* gene with regard to the mean of heterozigosity in the neutral *loci* indicates that this gene has undergone strong selection in Equatorial Guinea. The higher mean of *He* around double mutant than the mean of *He* around triple mutant is consistent with a model of positive directional selection. The Fst values at STR *loci* linked to *Pfdhfr* gene were higher when compared with mean Fst at neutral *loci*, which supports this hypothesis. However, no significant LD values were found between flanking genes of interest.

According to the results herein presented, SFX resistance seems to have appeared more recently than PYR resistance in mainland Equatorial Guinea. Indeed, only mutations at codons A437G and A581G, from the *Pfdhps* polymorphic sites surveyed showed high prevalence. Point mutations at S436A and K540E codons were rarely seen. A wide diversity of haplotypes was detected, being the majority unique haplotypes, which is consistent with independent origins for those alleles. The most prevalent haplotype match with AGK1/SGK1 lineages of West African origin and a few others (436A:437G:540K/ 436S:437G:540 K) with probable independent origin. The double mutant lineage identified as SGE1 (436S:437G:540E), originated in East Africa [28], was not detected in this study. As occurred with PYR resistance, the resistance to SFX seems to have been established earlier in Ngonamanga, where the prevalence of triple mutants is higher than in Miyobo.

When double mutants (*He*=0.81) were compared with triple mutants (*He*=0.67), a reduction in the heterozigosity was seen. However the values found are remarkably higher when comparing to those found for the PYR resistance (mean *He*=0.22). These differences may be due to the presence of multiple lineages occurring within individual populations. Also, significant LD values were found between flanking *Pfdhps* gene, involving *loci* 0.8 kb/4.3 kb and 4.3 kb/7.7Kb. Overall, these results might be suggestive of soft selective sweep, where multiple lineages are superimposed within a single population causing higher *He* values than in populations where a single lineage is present [63].

The results gathered in this study suggests that the PYR resistance has been established for a while in mainland Equatorial Guinea leaving selection signatures as the decrease in genetic diversity and an increased genetic differentiation at the *loci* around *Pfdhfr* gene. In addition, the impact on genetic diversity was less clear at the *loci* flanking *Pfdhps*, with only evidence of a soft selective sweep effect. This agrees with a more recent introduction of resistance to SFX in Equatorial Guinea, which is in agreement with results obtained in a recent study [64].

Conclusions

CQ is no longer recommended for malaria treatment in Equatorial Guinea but SP remains in use in combination with artesunate and is the only drug recommended for intermittent preventive therapy in pregnancy [65]. Prevalence of infection in the mainland region, where most of the country's population live, remains high despite the efforts undertaken to control malaria transmission mainly on the island of Bioko [10,34]. A close and continuous monitoring of point mutations frequency in the two genes associated with SP resistance, *Pfdhfr* and *Pfdhps*, is essential since there is the danger of an eventual reduction in the efficacy of SP combined therapy.

Additional files

Additional file 1: Prevalence of *Plasmodium* infections in humans, in two villages of mainland Equatorial Guinea. n: sample size; F: *P. falciparum*; M: *P. malariae*; O: *P. ovale;* V: *P. vivax*; F+M: mixed infection by *P. falciparum* and *P. malariae;* F+O: mixed infection by *P. falciparum* and *P. ovale*; F+V: mixed infection by *P. falciparum* and *P. vivax;* F+M+O: mixed infection by *P. falciparum*, *P. malariae* and *P. ovale*; F+M+V: mixed infection by *P. falciparum*, *P. malariae* and *P. vivax*.

Additional file 2: Prevalence of *Plasmodium* infections in mosquitoes, in two villages of mainland Equatorial Guinea. n: sample size; F: *P. falciparum*; M: *P. malariae*; O: *P. ovale;* V: *P. vivax*; F+M: mixed infection by *P. falciparum* and *P. malariae;* F+O: mixed infection by *P. falciparum* and *P. ovale*; F+V: mixed infection by *P. falciparum* and *P. vivax;* F+M+O: mixed infection by *P. falciparum*, *P. malariae* and *P. ovale*; F+M+V: mixed infection by *P. falciparum*, *P. malariae* and *P. vivax.*

Additional file 3: Characterization of mutations in *Pfcrt, Pfmdr1*, *Pfdhps* and *Pfdhfr* genes, in humans and mosquitoes.

Additional file 4: *Pfdhfr* point mutations and their respective STR haplotypes in allele size.

Additional file 5: *Pfdhps* point mutations and their respective STR haplotypes in allele size.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CM and VG carried out the laboratory analysis. JC carried out the sampling and field data collection. PS, PB, BdS, VER, AB and JC participated in the analysis and interpretation of data and helped to draft the manuscript. CM and APA drafted the paper. APA designed the study and participated in the analysis and interpretation of data. All authors read and approved the final manuscript.

Acknowledgements
We thank all families who participated in this study. We thank researchers and technicians from the National Malaria Control Program of the Equatorial Guinea's Ministry of Health and Social Welfare and "Centro de Referencia para el Control de Endemias" (Instituto de Salud Carlos III, Equatorial Guinea). This study was supported by PEst-OE/SAU/LA0018/2011 - Proj. Estratégico LA0018 2011/2012 (http://cmdt.ihmt.unl.pt/index.php/pt/) and PTDC/SAU-EPI/113326/2009, "Fundacão para a Ciência e Tecnologia/Ministério da Educação e Ciência", FCT/MEC (http://alfa.fct.mctes.pt/index.phtml.pt), Portugal and by "Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación", Madrid, Spain. C. Mendes and P. Salgueiro hold FCT grants (SRFH/BD/41473/2007 and SFRH/BPD/72532/2010, respectively).

Author details
[1]Centro de Malária e outras Doenças Tropicais, Unidade de Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal. [2]Centro Nacional de Medicina Tropical, Instituto de Salud Carlos III, Madrid, Spain. [3]Centro de Malária e outras Doenças Tropicais, Unidade de Saúde Internacional, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal.

Received: 26 November 2012 Accepted: 15 March 2013
Published: 27 March 2013

References
1. WHO: *World malaria report 2011*. Geneva: World Health Organization; 2011. http://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf.
2. Bruce MC, Donnelly CA, Alpers MP, Galinski MR, Barnwell JW, Walliker D, Day KP: **Cross-species interactions between malaria parasites in humans.** *Science* 2000, **287:**845–848.
3. Arez AP, Pinto J, Palsson K, Snounou G, Jaensen TGT, Rosário V: **Transmission of mixed Plasmodium species and *Plasmodium falciparum* genotypes.** *Am J Trop Med Hyg* 2003, **68:**161–198.
4. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, do Rosário VE, Benito A, Berzosa P, Arez AP: **Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea).** *PLoS Negl Trop Dis* 2011, **5:**e1192.
5. Mharakurwa S, Kumwenda T, Mkulama MAP, Musapa M, Chishimba S, Shiff CJ, Sullivan DJ, Thuma PE, Liu K, Agre P: **Malaria antifolate resistance with contrasting *Plasmodium falciparum* dihydrofolate reductase (DHFR) polymorphisms in humans and Anopheles mosquitoes.** *Proc Natl Acad Sci USA* 2011, **108:**18796–18801.
6. Arez AP, do Rosário VE: **The relevance of molecular markers in the analysis of malaria parasite populations.** *Transbound Emerg Dis* 2008, **55:**226–232.
7. Kiwanuka GN: **Genetic diversity in *Plasmodium falciparum* merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007.** *J Vector Borne Dis* 2009, **46:**1–12.
8. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP: **Microsatellite markers reveal a spectrum of population structures in the malaria parasite *P. falciparum*.** *Mol Biol Evol* 2000, **17:**1467–1482.
9. Pardo G, Descalzo MA, Molina L, Custodio E, Lwanga M, Mangue C, Obono J, Nchama A, Roche J, Benito A, Cano J: **Impact of different strategies to control *Plasmodium* infection and anaemia on the island of Bioko (Equatorial Guinea).** *Malar J* 2006, **5:**10.
10. Kleinschmidt I, Schwabe C, Benavente L, Torrez M, Ridl FC, Segura JL, Ehmer P, Nchama GN: **Marked increase in child survival after four years of intensive malaria control.** *Am J Trop Med Hyg* 2009, **80:**882–888.
11. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naudé B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Wellems TE: **Mutations in the *P. falciparum* digestive vacuole transmembrane protein Pfcrt and evidence for their role in Chloroquine resistance.** *Mol Cell* 2000, **6:**861–871.
12. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF: **Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*.** *Nature* 2000, **403:**906–909.
13. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ: **Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*.** *Proc Natl Acad Sci USA* 1988, **85:**9109–9113.
14. Brooks DR, Wang P, Read M, Watkins WM, Sims PF, Hyde JE: **Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine.** *Eur J Biochem* 1994, **224:**397–405.
15. Roche J, Guerra-Neira A, Raso J, Benito A: **Surveillance of in vivo resistance of *Plasmodium falciparum* to antimalarial drugs from 1992 to 1999 in Malabo (Equatorial Guinea).** *Am J Trop Med Hyg* 2003, **68:**598–601.
16. Charle P, Berzosa P, Descalzo MA, de Lucio A, Raso J, Obono J, Lwanga M, Nlang N, Nchama A, Mangue C, Micha A, Nsee N, Mesie R, Benito A, Roche J: **Efficacy of artesunate + sulphadoxine-pyrimethamine (AS + SP) and amodiaquine + sulphadoxine-pyrimethamine (AQ+ SP) for uncomplicated falciparum malaria in Equatorial Guinea (Central Africa).** *J Trop Med* 2009, **2009:**781865.
17. van Eijk AMV, Hill J, Alegana VA, Kirui V, Gething PW, Kuile FO, Snow RW: **Coverage of malaria protection in pregnant women in sub-Saharan Africa: a synthesis and analysis of national survey data.** *Lancet Infect Dis* 2011, **11:**190–207.
18. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE: **Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa.** *Am J Trop Med Hyg* 1995, **52:**565–568.
19. Arez AP, Lopes D, Pinto J, Franco AS, Snounou G, do Rosário VE: ***Plasmodium* sp.: Optimal protocols for PCR detection of low parasite numbers from mosquito (*Anopheles* sp.) samples.** *Exp Parasitol* 2000, **94:**269–272.
20. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN: **Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of high prevalence of mixed infections.** *Mol Biochem Parasitol* 1993, **58:**283–292.
21. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S: **Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand.** *Trans R Soc Trop Med Hyg* 1999, **93:**369–374.
22. *PCR-ASRA Protocols for Plasmodium falciparum Drug-Resistance Mutation Analysis.* 2002. http://medschool.umaryland.edu/cvd/plowe.html.
23. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP: **Twelve microsatellite markers characterization of *P. falciparum* from finger-prick blood samples.** *Parasitology* 1999, **119:**113–125.
24. Conway DJ, Machado RL, Singh B, Dessert P, Mikes ZS, Povoa MM, Oduola AM, Roper C: **Extreme geographical fixation of variation in the *Plasmodium falciparum* gamete surface protein gene Pfs48/45 compared with microsatellite loci.** *Mol Biochem Parasitol* 2001, **115:**145–156.
25. Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, Chandramohan D, Sharp B: **Antifolate antimalarial resistance in southeast Africa: a population-based analysis.** *Lancet* 2003, **361:**1174–1181.
26. Ndiaye D, Daily JP, Sarr O, Ndir O, Gaye O, Mboup S, Roper C, Wirth DF: **Defining the origin of *Plasmodium falciparum* resistant dhfr isolates in Senegal.** *Acta Trop* 2006, **99:**106–111.
27. Salgueiro P, Vicente JL, Ferreira C, Teófilo V, Galvão A, Do Rosário VE, Cravo P, Pinto J: **Tracing the origins and signatures of selection of antifolate resistance in island populations of *Plasmodium falciparum*.** *BMC Infect Dis* 2010, **10:**163.
28. Pearce RJ, Pota H, Evehe MS, Bâ e-H, Mombo-Ngoma G, Malisa AL, Ord R, Inojosa W, Matondo A, Diallo DA, Mbacham W, van den Broek IV, Swarthout

TD, Getachew A, Dejene S, Grobusch MP, Njie F, Dunyo S, Kweku M, Owusu-Agyei S, Chandramohan D, Bonnet M, Guthmann JP, Clarke S, Barnes KI, Streat E, Katokele ST, Uusiku P, Agboghoroma CO, Elegba OY, Cissé B, A-Elbasit IE, Giha HA, Kachur SP, Lynch C, Rwakimari JB, Chanda P, Hawela M, Sharp B, Naidoo I, Roper C: **Multiple origins and regional dispersal of resistant dhps in African *Plasmodium falciparum* malaria.** *PLoS Med* 2009, **6:**e1000055.

29. Uitenbroek DG: *SISA Binomial.* 1997. http://www.quantitativeskills.com/sisa/.
30. Goudet J: **FSTAT (version 1.2): A program to estimate and test gene diversities and fixation indices.** *J Hered* 2002, **86:**485–486.
31. Raymond M, Rousset F: **GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenism.** *J Hered* 1995, **86:**248–249.
32. Rice WR: **Analyzing tables of statistical tests.** *Evolution* 1989, **43:**223–225.
33. Kleinschmidt I, Torrez M, Schwabe C, Benavente L, Seocharan I, Jituboh D, Nseng G, Sharp B: **Factors influencing the effectiveness of malaria control in Bioko Island, Equatorial Guinea.** *Am J Trop Med Hyg* 2007, **76:**1027–1032.
34. Overgaard HJ, Reddy VP, Abaga S, Matias A, Reddy MR, Kulkarni V, Schwabe C, Segura L, Kleinschmidt I, Slotman MA: **Malaria transmission after five years of vector control on Bioko Island, Equatorial Guinea.** *Parasit Vectors* 2012, **5:**253.
35. Molineaux L, Storey J, Cohen JE, Thomas A: **A longitudinal study of human malaria in the West *African Savanna in th*e absence of control measures: relationships between different Plasmodium species, in particular *P. falciparum* and *P. malariae*.** *Am J Trop Med Hyg* 1980, **29:**725–737.
36. Richie TL: **Interactions between malaria parasites infecting the same vertebrate host.** *Parasitology* 1988, **96:**607–639.
37. Boudin C, Robert V, Verhave JP, Carnevale P, Ambroise-Thomas P: ***Plasmodium falciparum* and *P. malariae* epidemiology in a West African village.** *Bull World Health Organ* 1991, **69:**199–205.
38. Marques PX, Saúte F, Pinto VV, Cardoso S, Pinto J, Alonso PL, do Rosário VE, Arez AP: **Plasmodium species mixed infections in two ecologically different areas of Manhiça District, Mozambique.** *Int J Biol Sci* 2005, **1:**96–102.
39. Bruce MC, Macheso A, Kelly-Hope LA, Nkhoma S, McConnachie A, Molyneux ME: **Effect of transmission setting and mixed species infections on clinical measures of malaria in Malawi.** *PLoS One* 2008, **3:**e2775.
40. Babiker HA, Satti G, Walliker D: **Genetic changes in the population of *Plasmodium falciparum* in a Sudanese village over a three-year period.** *Am J Trop Med Hyg* 1995, **53:**7–15.
41. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, Conway DJ: **Population genetic structure of *Plasmodium falciparum* across a region of diverse endemicity in West Africa.** *Malar J* 2012, **11:**223.
42. Cano J, Berzosa P, de Lucio A, Descalzo MA, Bobuakasi L, Nzambo S, Ondo M, Buatiche JN, Nseng G, Benito A: **Transmission of malaria and genotypic variability of *Plasmodium falciparum* on the Island of Annobon (Equatorial Guinea).** *Malar J* 2007, **6:**141.
43. Sá JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE: **Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine.** *Proc Natl Acad Sci USA* 2009, **106:**18883–18889.
44. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimdé AA, Kouriba B, Taylor TE, Plowe CV: **Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi.** *J Infect Dis* 2003, **187:**1870–1875.
45. Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K, Borrmann S, Mackinnon M, Nzila A: **Chloroquine resistance before and after its withdrawal in Kenya.** *Malar J* 2009, **8:**106.
46. Wang X, Mu J, Li G, Chen P, Guo X, Fu L, Chen L, Su X, Wellems TE: **Decreased prevalence of the *Plasmodium falciparum* chloroquine resistance transporter 76 T marker associated with cessation of chloroquine use against *P. falciparum* malaria in Hainan, People's Republic of China.** *Am J Trop Med Hyg* 2005, **72:**410–414.
47. Fançony C, Gamboa D, Sebastião Y, Hallett R, Sutherland C, Sousa-Figueiredo JC, Nery SV: **Various pfcrt and pfmdr1 genotypes of *Plasmodium falciparum* cocirculate with *P. malariae*, *P. ovale* spp., and *P. vivax* in Northern Angola.** *Antimicrob Agents Chemother* 2012, **56:**5271–5277.
48. Amor A, Toro C, Fernández-Martínez A, Baquero M, Benito A, Berzosa P: **Molecular markers in *Plasmodium falciparum* linked to resistance to anti-malarial drugs in samples imported from Africa over an eight-year period (2002-2010): impact of the introduction of artemisinin combination therapy.** *Malar J* 2012, **11:**100.
49. Plowe CV, Wellems TE: **Molecular approaches to the spreading problem of drug resistant malaria.** *Adv Exp Med Biol* 1995, **390:**197–209.
50. McCollum AM, Poe AC, Hamel M, Huber C, Zhou Z, Shi YP, Ouma P, Vulule J, Bloland P, Slutsker L, Barnwell JW, Udhayakumar V, Escalante AA: **Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel dhfr alleles.** *J Infect Dis* 2006, **194:**189–197.
51. Trigg JK, Mbwana H, Chambo O, Hills E, Watkins W, Curtis CF: **Resistance to pyrimethamine/sulfadoxine in *Plasmodium falciparum* in 12 villages in northeast Tanzania and a test of chlorproguanil/dapsone.** *Acta Trop* 1997, **63:**185–189.
52. Tahar R, Basco LK: **Molecular epidemiology of malaria in Cameroon. XXII. Geographic mapping and distribution of *Plasmodium falciparum* dihydrofolate reductase (dhfr) mutant alleles.** *Am J Trop Med Hyg* 2006, **75:**396–401.
53. Aubouy A, Jafari S, Huart V, Migot-Nabias F, Mayombo J, Durand R, Bakary M, Le Bras J, Deloron P: **DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with in vitro activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy.** *J Antimicrob Chemother* 2003, **52:**43–49.
54. Mawili-Mboumba DP, Ekala MT, Lekoulou F, Ntoumi F: **Molecular analysis of DHFR and DHPS genes in *P. falciparum* clinical isolates from the Haut-Ogooué region in Gabon.** *Acta Trop* 2001, **78:**231–240.
55. Alker AP, Kazadi WM, Kutelemeni AK, Bloland PB, Tshefu AK, Meshnick SR: **dhfr and dhps genotype and sulfadoxine-pyrimethamine treatment failure in children with falciparum malaria in the Democratic Republic of Congo.** *Trop Med Int Health* 2008, **13:**1384–1391.
56. Spalding MD, Eyase FL, Akala HM, Bedno SA, Prigge ST, Coldren RL, Moss WJ, Waters NC: **Increased prevalence of the pfdhfr/phdhps quintuple mutant and rapid emergence of pfdhps resistance mutations at codons 581 and 613 in Kisumu, Kenya.** *Malar J* 2010, **9:**338.
57. Kublin JG, Dzinjalamala FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, Mukadam RA, Rogerson SJ, Lescano AG, Molyneux ME, Winstanley PA, Chimpeni P, Taylor TE, Plowe CV: **Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria.** *J Infect Dis* 2002, **185:**380–388.
58. Talisuna AO, Nalunkuma-Kazibwe A, Langi P, Mutabingwa TK, Watkins WW, Van Marck E, Egwang TG, D'Alessandro U: **Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated falciparum malaria.** *Infect Genet Evol* 2004, **4:**321–327.
59. Alam MT, de Souza DK, Vinayak S, Griffing SM, Poe AC, Duah NO, Ghansah A, Asamoa K, Slutsker L, Wilson MD, Barnwell JW, Udhayakumar V, Koram K: **Selective sweeps and genetic lineages of *Plasmodium falciparum* drug-resistance in Ghana.** *J Infect Dis* 2011, **203:**220–227.
60. Maïga O, Djimdé AA, Hubert V, Renard E, Aubouy A, Kironde F, Nsimba B, Koram K, Doumbo OK, Le Bras J, Clain J: **A shared Asian origin of the triple-mutant dhfr allele in *Plasmodium falciparum* from sites across Africa.** *J Infect Dis* 2007, **196:**165–172.
61. Malisa AL, Pearce RJ, Abdulla S, Mshinda H, Kachur PS, Bloland P, Roper C: **Drug coverage in treatment of malaria and the consequences for resistance evolution - evidence from the use of sulphadoxine/pyrimethamine.** *Malar J* 2010, **9:**190.
62. Escalante AA, Cornejo OE, Rojas A, Udhayakumar V, Lal AA: **Assessing the effect of natural selection in malaria parasites.** *Trends Parasitol* 2004, **20:**388–395.
63. Nair S, Nash D, Sudimack D, Jaidee A, Barends M, Uhlemann AC, Krishna S, Nosten F, Anderson TJ: **Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites.** *Mol Biol Evol* 2007, **24:**562–573.
64. Naidoo I, Roper C: **Following the path of most resistance: dhps K540E dispersal in African *Plasmodium falciparum*.** *Trends Parasitol* 2010, **26:**447–456.
65. Kleinschmidt I, Sharp B, Benavente LE, Schwabe C, Torrez M, Kuklinski J, Morris N, Raman J, Carter J: **Reduction in infection with *Plasmodium falciparum* one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea.** *Am J Trop Med Hyg* 2006, **74:**972–978.

doi:10.1186/1475-2875-12-114
Cite this article as: Mendes *et al.*: Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea. *Malaria Journal* 2013 **12**:114.

Chapter 2

Additional file 1

Additional file 1. Prevalence of *Plasmodium* infections in humans, in two villages of mainland Equatorial Guinea.

	Village	Ngonamanga			Miyobo			Total	
	Season	**Dry**	**Rainy**	**Total**	**Dry**	**Rainy**	**Total**	**n**	**%**
	n	40	26	53	34	43	44	**97**	**-**
	n of positive samples	26 (65%)	17 (65.4%)	43 (81.1%)	25 (73.5%)	30 (68.8%)	41 (93.2%)	**84**	**86.6**
Single infection	**F**	24	15	68	18	17	52	62	63.9
	M	0	0	0	2	0	4	2	2.1
	O	0	0	0	0	0	0	0	0.0
	V	0	0	0	2	0	4	2	2.1
Mixed infection	**F + M**	2	0	4	1	3	7	5	5.2
	F + O	0	2	4	2	2	4	4	4.1
	F + V	0	0	0	2	2	11	5	5.2
	F + M + O	0	0	0	0	3	7	3	3.1
	F + M + V	0	0	0	0	1	2	1	1.0
	Total	2	2	8	5	11	32	18	18.6
Overall infection F		**26**	**17**	**100**	**23**	**28**	**90**	**80**	**95.2**
Overall infection M		**2**	**0**	**5**	**3**	**7**	**22**	**11**	**13.1**
Overall infection V		**0**	**0**	**0**	**4**	**3**	**20**	**8**	**9.5**
Overall infection O		**0**	**2**	**5**	**2**	**5**	**12**	**7**	**8.3**

n: sample size; **F:** *P. falciparum*; **M:** *P. malariae*; **O:** *P. ovale;* **V:** *P. vivax*; **F+M:** mixed infection by *P. falciparum* and *P. malariae;* **F+O:** mixed infection by *P. falciparum* and *P. ovale*; **F+V:** mixed infection by *P. falciparum* and *P. vivax;* **F+M+O:** mixed infection by *P. falciparum*, *P. malariae* and *P. ovale*; **F+M+V:** mixed infection by *P. falciparum*, *P. malariae* and *P. vivax.*

104

Additional file 2

Additional file 2. Prevalence of *Plasmodium* infections in mosquitoes, in two villages of mainland Equatorial Guinea.

Village		**Ngonamanga**				**Miyobo**				**Total**	
Season		**Dry**		**Rainy**		**Dry**		**Rainy**			
		Head +Torax	**Abdomen**	**Head +Torax**	**Abdomen**	**Head +Torax**	**Abdómen**	**Head +Torax**	**Abdomen**	**n**	**%**
n		249	249	61	61	386	386	123	123	1638	%
n of positive samples		46	54	9	14	31	55	24	42	**275**	**16.8**
		18.50%	**21.60%**	**14.80%**	**23%**	**8.03%**	**14.25%**	**19.51%**	**34.15%**		
Single infection	**F**	39	47	7	14	29	42	24	35	237	14.5
	V	4	4	2	0	2	10	0	0	22	1.3
	M	1	0	0	0	0	2	0	1	4	0.2
	O	0	0	0	0	0	0	0	0	0	0.0
Mixed infection	**F + V**	1	0	0	0	0	1	0	0	2	0.1
	F + M	0	2	0	0	0	0	0	4	6	0.4
	F + O	0	1	0	0	0	0	0	2	3	0.2
	F + M + O	1	0	0	0	0	0	0	0	1	0.1
Overall infection F		**41**	**50**	**7**	**14**	**29**	**43**	**24**	**41**	**249**	**90.5**
Overall infection V		**5**	**4**	**2**	**0**	**2**	**11**	**0**	**0**	**24**	**8.7**
Overall infection M		**2**	**2**	**0**	**0**	**0**	**2**	**0**	**5**	**11**	**4.0**
Overall infection O		**1**	**1**	**0**	**0**	**0**	**0**	**0**	**2**	**4**	**1.5**

n: sample size; **F:** *P. falciparum*; **M:** *P. malariae*; **O:** *P. ovale;* **V:** *P. vivax*; **F+M:** mixed infection by *P. falciparum* and *P. malariae;* **F+O:** mixed infection by *P. falciparum* and *P. ovale*; **F+V:** mixed infection by *P. falciparum* and *P. vivax;* **F+M+O:** mixed infection by *P. falciparum*. *P. malariae* and *P. ovale*; **F+M+V:** mixed infection by *P. falciparum*. *P. malariae* and *P. vivax*

Additional file 3

Additional file 3. Characterization of mutations in *Pfcrt*, *Pfmdr1*, *Pfdhps* and *Pfdhfr* genes, in humans and mosquitoes.

			Pfcrt		***Pfmdr1***		***Pfdhfr***				***Pfdhps***			
			N75E	**K76T**	**N86Y**	**D1246Y**	**N51I**	**C59R**	**S108N**	**I164L**	**S436A**	**A437G**	**K540E**	**A581G**
Humans	**Ngonamanga**	**n**	113	113	113	113	113	113	113	113	113	113	113	113
		wild type	0.33	0.42	0.15	1	0.09	0.8	0.05	0.98	0.5	0.25	98	0
		mutant	0.67	0.58	0.85	0	0.91	0.92	0.95	0.02	0.5	0.75	0.02	1
	Miyobo	**n**	194	212	215	184	199	195	195	213	212	249	190	190
		wild type	0.55	0.40	0.29	0.98	0.44	0.25	0.11	0.80	0.73	0.48	1	0
		mutant	0.45	0.60	0.71	0.02	0.56	0.75	0.89	0.20	0.27	0.52	0	1
	Dry season	**n**	168	174	185	168	172	168	168	181	188	205	167	167
		wild type	0.46	0.32	0.24	0.99	0.19	0.13	0.04	0.92	0.59	0.39	1	0
		mutant	0.54	0.68	0.76	0.01	0.81	0.87	0.96	0.08	0.41	0.61	0	1
	Wet season	**n**	139	151	143	129	140	140	140	145	137	157	136	136
		wild type	0.48	0.51	0.25	0.98	0.47	0.25	0.15	0.79	0.74	0.43	0.99	0
		mutant	0.52	0.49	0.75	0.02	0.53	0.75	0.85	0.21	0.26	0.57	0.01	1
	Total	**n**	307	325	328	297	312	308	308	326	325	362	303	303
		wild type	0.47	0.41	0.24	0.99	0.31	0.19	0.09	0.86	0.65	0.41	0.99	0
		mutant	0.53	0.59	0.76	0.01	0.69	0.81	0.91	0.14	0.35	0.59	0.01	1

Mosquitoes	**Ngonamanga**	**n**	12	33	25	26	35	38	32	32	28	30	33	30
		wild type	0.25	0.31	0.68	0.96	0.26	0.29	0	1	0.89	0.2	0.91	0
		mutant	0.75	0.69	0.32	0.04	0.74	71	1	0	0.11	0.8	0.09	1
	Miyobo	**n**	29	43	41	53	17	16	51	26	54	57	56	43
		wild type	0.66	0.72	0.27	1	0.06	0.06	0.1	1	0.85	0.26	0.96	0
		mutant	0.34	0.28	0.73	0	0.94	0.94	0.90	0	0.15	0.74	0.04	1
	Dry season	**n**	17	26	27	34	33	35	31	31	35	40	39	37
		wild type	0.41	0.46	0.37	0.97	0.27	0.26	0.10	1	0.91	0.15	0.95	0
		mutant	0.59	54	0.63	0.03	0.73	0.74	0.9	0	0.09	0.85	0.05	1
	Wet season	**n**	24	49	39	45	19	19	52	27	47	47	50	36
		wild type	0.63	0.59	0.46	1	0.05	0.16	0.04	1	0.83	0.32	0.94	0
		mutant	0.38	0.41	54	0	0.95	0.84	0.96	0	0.17	0.68	0.06	1
	Total	**n**	41	75	66	79	52	54	83	58	82	87	89	73
		wild type	0.54	0.55	0.42	0.99	0.19	0.22	0.06	1	0.87	0.24	0.94	0
		mutant	0.46	0.45	0.58	0.01	0.81	0.78	0.94	0	0.13	0.76	0.06	1

Additional file 4

Additional file 4. *Pfdhfr* point mutations and their respective STR haplotypes in allele size.

Villages	Haplotype	Point mutation	Allele size (bp)			N
			locus 0.8kb	*locus* 4.3kb	*locus* 7.7kb	
	K1	**R59/N108**	113	183	210	
Ngonamanga	**H1**	**N108**	113	183	210	1
	H2	**I51/R59/N108**	113	179	210	1
	H3	**I51/R59/N108**	113	183	210	32
	H4	**I51/R59/N108**	113	183	214	1
Miyobo	**H1**	**N108**	113	183	210	2
	H3	**I51/R59/N108**	113	183	210	6
	H5	**R59**	113	183	210	1
	H6	**N108**	107	183	200	1
	H7	**I51/N108**	117	183	210	1
	H8	**I51/N108**	113	183	210	1
	H9	**R59/N108**	113	183	210	10

Additional file 5

Additional file 5. *Pfdhps* point mutations and their respective STR haplotypes in allele size.

Village	Haplotype	Point mutation	Allele size (bp)			N
			***locus* 0.8kb**	***locus* 4.3kb**	***locus* 7.7kb**	
	K1	**A437G**	131	103	108	
Ngonamanga	**H1**	**A436/G581**	117	105	124	1
	H2	**A436/G581**	117	109	118	1
	H3	**G437/G581**	113	107	110	1
	H4	**G437/G581**	115	103	120	1
	H5	**G437/G581**	121	109	112	1
	H6	**G437/G581**	123	107	110	1
	H7	**G437/G581**	131	117	126	2
	H8	**A436/G437/G581**	121	109	112	4
	H9	**A436/G437/G581**	133	103	120	1
Miyobo	**H10**	**G581**	117	109	124	1
	H11	**G581**	117	105	126	2
	H12	**G581**	117	117	126	1
	H13	**G581**	121	111	118	1
	H14	**A436/G581**	117	109	126	1
	H15	**A436/G581**	123	103	126	1
	H16	**G437/G581**	117	103	138	1
	H17	**G437/G581**	117	105	120	2
	H18	**G437/G581**	117	105	138	1
	H19	**G437/G581**	121	107	110	1
	H20	**G437/G581**	121	107	114	2

	H5	**G437/G581**	121	109	112	1
	H21	**G437/G581**	123	109	112	1
	H22	**G437/G581**	123	107	110	3
	H23	**G437/G581**	125	107	114	1
	H24	**G437/G581**	135	103	120	1
	H25	**A436/G437/G581**	121	107	114	1

Chapter 3- Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – Molecular evidences from the African West Coast (Angola and Equatorial Guinea)

This chapter was published as a research paper:

Mendes C., Dias F., Figueiredo J., Mora V.G., Cano J., de Sousa B., do Rosário V.E., Benito A., Berzosa P., Arez A.P. (2011) Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLos Neglected Tropical Disease* 5(6):e1192

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

Duffy Negative Antigen Is No Longer a Barrier to *Plasmodium vivax* – Molecular Evidences from the African West Coast (Angola and Equatorial Guinea)

Cristina Mendes[1], Fernanda Dias[1,2], Joana Figueiredo[1], Vicenta Gonzalez Mora[3], Jorge Cano[3], Bruno de Sousa[4], Virgílio E. do Rosário[1], Agustin Benito[3], Pedro Berzosa[3], Ana Paula Arez[1]*

1 Centro de Malária e outras Doenças Tropicais, Unidade de Parasitologia, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal, **2** Departamento de Medicina Interna, Faculdade de Medicina, Universidade Agostinho Neto, Luanda, Angola, **3** Centro Nacional de Medicina Tropical, Instituto de Salud Carlos III, Madrid, Spain, **4** Centro de Malária e outras Doenças Tropicais, Unidade de Saúde Internacional, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal

Abstract

Background: *Plasmodium vivax* shows a small prevalence in West and Central Africa due to the high prevalence of Duffy negative people. However, Duffy negative individuals infected with *P. vivax* have been reported in areas of high prevalence of Duffy positive people who may serve as supply of *P. vivax* strains able to invade Duffy negative erythrocytes. We investigated the presence of *P. vivax* in two West African countries, using blood samples and mosquitoes collected during two on-going studies.

Methodology/Findings: Blood samples from a total of 995 individuals were collected in seven villages in Angola and Equatorial Guinea, and 820 *Anopheles* mosquitoes were collected in Equatorial Guinea. Identification of the *Plasmodium* species was achieved by nested PCR amplification of the small-subunit rRNA genes; *P. vivax* was further characterized by *csp* gene analysis. Positive *P. vivax*-human isolates were genotyped for the Duffy blood group through the analysis of the *DARC* gene. Fifteen Duffy-negative individuals, 8 from Equatorial Guinea (out of 97) and 7 from Angola (out of 898), were infected with two different strains of *P. vivax* (VK210 and VK247).

Conclusions: In this study we demonstrated that *P. vivax* infections were found both in humans and mosquitoes, which means that active transmission is occurring. Given the high prevalence of infection in mosquitoes, we may speculate that this hypnozoite-forming species at liver may not be detected by the peripheral blood samples analysis. Also, this is the first report of Duffy negative individuals infected with two different strains of *P. vivax* (VK247 and classic strains) in Angola and Equatorial Guinea. This finding reinforces the idea that this parasite is able to use receptors other than Duffy to invade erythrocytes, which may have an enormous impact in *P. vivax* current distribution.

Citation: Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, et al. (2011) Duffy Negative Antigen Is No Longer a Barrier to *Plasmodium vivax* – Molecular Evidences from the African West Coast (Angola and Equatorial Guinea). PLoS Negl Trop Dis 5(6): e1192. doi:10.1371/journal.pntd.0001192

Editor: Carlos Franco-Paredes, Emory University, United States of America

Received January 7, 2011; **Accepted** April 17, 2011; **Published** June 21, 2011

Copyright: © 2011 Mendes et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by "Faculdade de Medicina Américo Boavida", Angola; by the "Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación", Madrid, Spain; by "Financiamento Programático do Laboratório Associado CMDT.LA/IHMT" (http://www.ihmt.unl.pt/) and PTDC/SAU-EPI/113326/2009, "Fundacão para a Ciência e Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior", FCT/MCTES (http://alfa.fct.mctes.pt/index.phtml.pt), Portugal. Cristina Mendes holds a FCT grant (SRFH/BD/41473/2007). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: aparez@ihmt.unl.pt

Introduction

Plasmodium vivax has been neglected by the scientific community since it has been seen as a "benign" parasite. Nowadays this scenario has changed and the infection caused by *P. vivax* gained higher importance, firstly because it has a very wide distribution, being found both in tropical and subtropical areas [1,2,3]; and secondly because of the high number of clinical cases reported, ranging from 70 million to 300 million [2,4,5]. Although clinical symptoms are usually considered as not severe, some reports documented cases of severe disease and even death [6,7,8,9].

This parasite has traditionally shown a small prevalence in West and Central Africa, attributed to the high prevalence of Duffy negative people [Fy(a−b−)] who are described as being resistant to *P. vivax* infection [10,11]. Culleton et al. [12] performed a study including nine endemic countries of West and Central Africa using a high sensitive PCR-based protocol for the detection and identification of *Plasmodium* species reporting only one case out of 2588 individuals infected with *P. vivax* - one Duffy-positive individual from São Tomé. Although the exact prevalence of *P. vivax* in Africa is unknown, this parasite tends to be endemic in countries of East Africa, like Sudan, Somalia and Ethiopia, where the majority of the population is Duffy-positive.

The Duffy antigen, also called Duffy antigen receptor for chemokines (DARC), is a multimeric red cell membrane protein organized into seven transmembrane domains, and it is the unique

Author Summary

Recent reports of *Plasmodium vivax* infections, the most widely distributed species of human malaria, show that this parasite is evolving and adapting, becoming not only more aggressive but also more frequent in countries where it was not present in the past, becoming, therefore, a major source of concern. Thus, it is extremely important to perform new studies of its distribution in West and Central Africa, where there are few reports of its presence, due to the high prevalence of Duffy-negative individuals. The aim of this study was to investigate the presence of *P. vivax* in Angola and in Equatorial Guinea, using blood samples and mosquitoes. The results showed that *P. vivax* seems to be able to invade erythrocytes using receptors other than Duffy, and this new capacity is not exclusive to one strain of *P. vivax*, since we have found samples infected with two different strains: VK247 and classic. Additionally we demonstrated that the parasite has a greater distribution than previously thought, calling for a reevaluation of its worldwide distribution.

known erythrocyte receptor for *P. vivax* invasion. DARC-coding gene is polymorphic with multiple alleles as the codominant FY*A and FY*B, which encode for the two antigens – Fya and Fyb. Four genotypes are possible as a result of the combination of the major alleles, Fy(a+b+), Fy(a+b−), Fy(a−b+) and Fy(a−b−) [13,14,15]. The first three correspond to a Duffy-positive phenotype, mostly prevalent in Asian and in Caucasian populations and the last one correspond to the Duffy-negative phenotype, mainly prevalent in African people, who are consequently resistant to *P. vivax* infection. The Fy(a−b−) genotype results from a point mutation, -33T>C, in the promoter region of allele FY*B, in the GATA box region [13].

Recent data showed that Duffy binding protein, the main vaccine candidate for *P. vivax* [16,17], seems no longer to protect against *P. vivax* infection. Rosenberg [18] hypothesized that *P. vivax* could infect Duffy negative erythrocytes, since there were reports of European travellers and immigrants from West and Central Africa who were infected with *P. vivax* [19,20,21]. In fact, there are now other reports that seem to support this hypothesis [18].

In a case-control study conducted in Kenya, an East African country, with children with severe malaria caused by *Plasmodium falciparum*, it was found that there were children infected with *P. vivax* VK247 despite being Duffy-negative [22]. Similar results were found in the Amazon region in Brazil [23,24] and more recently in Madagascar [25]. These new data suggest that *P. vivax* may be evolving by using alternative receptors to bind and invade erythrocytes or it may be a "*vivax*-like" that do not require Duffy antigen for the invasion [26].

Currently, three different strains of *P. vivax* have been described – classic *P. vivax* (also called *P. vivax* VK 210), *P. vivax* VK 247 and *P. vivax-like*. These strains, although morphologically similar, differ in the central portion of circunsporozoite surface protein (*csp*), an abundant polypeptide present at the sporozoite surface [27]. The variant VK247 was first described by Rosenberg et al. [28] in isolates from Thailand and differs from the *P. vivax* classic in the nonapeptide repeat units of the central portion of CSP gene: ANGA(G/D)(N/D)QPG in *P. vivax* VK247 and GDRA(A/D)GQPA in *P. vivax* classic (described in [29]). Qari et al. [26] identified the strain *P. vivax-like*, characterised by having a 11-mer repeat sequence, APGNQ(E/G)GGAA in the central portion of the CSP gene.

With new cases of *P. vivax* infections appearing every day, especially in countries where this parasite has not been reported before, it becomes essential not to underestimate it, since *P. vivax* may be swiftly evolving and infecting people that were thought to be protected.

The aims of this study were to investigate the presence of *P. vivax* in Angola and in Equatorial Guinea, using blood samples and mosquitoes, and analyze the presence of *P. vivax* infection in Duffy-negative individuals.

Methods

Ethics statement

Each person (or parent) was informed of the nature and aims of the study and told that participation was voluntary and that they could withdraw from the study at any time. Blood samples were collected after informed consent from all donors (parents or guardians respond on behalf of children). In Equatorial Guinea, written consent was not obtainable because of the community-wide mistrust of signing any official forms and the low level of literacy in the population. Viewing this, written consent was only assented by population in case of the legal guardians of the recruited children and only non-documented oral consent was requested on adults. The study was approved by the Ethical Committee of the Equatorial Guinea's Ministry of Health and Social Welfare, the National Malaria Control Programme and the local health authorities from these villages, which accepted this constraint and did not find bio-ethical impediments to disallow the study. In Angola, written informed consent was obtained from each person (or parent/guardian) and the study was approved by the Ethical Committee of the Angola's Ministry of Health. Ethical clearance was also given by the Ethical Committees of IHMT and the ISCIII, according to EU norms.

Sampling

Blood samples were collected as part of two on-going studies in Angola and Equatorial Guinea (see figure 1).

Angola samples were collected in Gabela (10°S51′/14°E22′), Porto Amboim (10°S43′/13°E46′), Kissala-Sumbe (11°S12′/13°E50′), Praia-Sumbe (11°S12′/13°E50′) [Kuanza Sul province] and Funda (8°S50′/13°E33′) [Bengo province] between June 2006 and May 2007. In these two provinces malaria is mesoendemic stable and the climate is tropical, characterised by a wet and warm season, from September to April, and a dry and cold season, from May to August. In each village, blood samples were collected by fingerprick on filter paper, from asymptomatic children older than 2 months.

In Equatorial Guinea, blood samples and mosquito specimens were collected from 20 households in two different villages - Miyobo (1°N45′/10°E10′) in May and August of 2005 and Ngonamanga (2°N9′/9°E48′) in February and May of 2005. The two villages present different ecological characteristics: Miyobo is located in the interior of the country in a forested area, while Ngonamanga is a coastal area. In both, malaria is classified as hyperendemic, and it is possible to distinguish four seasons, two dry seasons from December to March and from July to September; and two wet seasons, one more intense from September to November and the other from March to the end of June. In each household, blood fed resting mosquitoes were collected early in the morning (5.00–7.00am), followed by blood sample collection by fingerprick from all inhabitants, during four consecutive days. Mosquitoes were kept in paper-cups corresponding to each house/room for 8 days to enable the development of oocysts from infections acquired the night prior to collection. Head/thorax and abdomen from each mosquito were kept separately for subsequent molecular processing.

Figure 1. Map of the five collection places in Equatorial Guinea and Angola. [Footnote: A- Equatorial Guinea; B- Angola; 1- Ngonamanga; 2- Miyobo; 3- Gabela; 4 – Porto Amboim and 5- Sumbe].
doi:10.1371/journal.pntd.0001192.g001

Sample collection and DNA extraction

Blood samples from a total of 995 individuals (898 from Angola and 97 from Equatorial Guinea) were collected by fingerprick on filter paper and stored at room temperature until DNA extraction, which was carried out using the chelex protocol as described by Plowe et al. [30].

DNA from the 819 mosquitoes captured in Equatorial Guinea was extracted using the chelex protocol described by Arez et al. [31]. DNA from portions head/thorax and abdomen of each mosquito was extracted separately.

Detection and identification of *Plasmodium* species

For all samples, detection of malaria infection and identification of *Plasmodium* species was made using nested-PCR amplification of the small subunit ribosomal RNA genes as described by Snounou et al. [32].

Genotyping of Pvcsp genes

In samples infected with *P. vivax*, parasite characterisation was carried out by analysis of the central region of the *Pvcsp* gene, following a slightly modified version of the protocol described by Alves et al. [33]. This was amplified in a MyCyclerTM Thermal cycler (Biorad), using the primers VivF 5′- TCCATCCTG-TTGGTGGACTT – 3′ and VivR 5′ – TCACAACGTTAAAT-ATGCCAG – 3′ with final reagent concentrations of 1× reaction buffer, 1 mM of $MgCl_2$, 100 µM of each dNTPs, 0.5 µM of each primer and 1 U/µl of Taq DNA Polymerase (Promega), in a total volume of 50 µl for each reaction. The PCR cycle conditions were: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 93°C for 1 minute, 60°C for 90 seconds and 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

In order to distinguish the three *P. vivax* strains (VK210, VK247 and *P. vivax-like*), restriction fragment length polymorphism (RFLP) analysis was performed using the restriction endonucleases (AluI and DpnI), following the recommended protocol (New England Biolabs, Ipswich, MA). PCR-RFLP products were run in a 2% agarose gel.

Genotyping of Duffy blood group

Duffy genotypes were also determined in *P. vivax* human isolates. To detect the point mutation -33T>C, which correspond to a Duffy-negative phenotype, the DARC gene promoter regions were amplified by PCR, followed by enzymatic restriction with StyI (New England Biolabs, Ipswich, MA) (adapted from [13]). Briefly, the PCR was performed using the primer P38 5′-

AGGCTTGTGCAGGCAGTG - 3′ and P39 5′- GGCATAGG-GATAAGGGACT - 3′, 0.5 pmol/µl of each, 1 mM of $MgCl_2$, 200 µM of dNTP's and 1 U/µl of Taq DNA Polymerase (Promega), in a total volume of 30 µl. Cycling parameters were as follows: 94°C for 5 minutes, pursued by 30 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

Endonuclease StyI was used for RFLP analysis of PCR products, according to the supplier's specifications (New England Biolabs, Ipswich, MA). Restriction fragments were separated on an 18% acrylamide/bis-acrylamide (39.5:1) gel and silver stained.

For confirmation, some samples were purified with the SureClean Kit (Bioline) according to manufacturer's recommendations and were sequenced in both directions by Macrogen, Korea.

Results

Detection and identification of *Plasmodium* species

The four species of *Plasmodium* were identified in both countries. *Plasmodium vivax* had not been previously described in the mainland of Equatorial Guinea.

Prevalence of infection in both blood samples and mosquitoes is presented in Table 1. Regarding the human host, overall prevalence of infection was much higher in Equatorial Guinea than in Angola (86.6% *versus* 28.9%, respectively), with *P. falciparum* showing the highest infection rate in both countries (95.2% in Equatorial Guinea and 97.9% in Angola). *Plasmodium vivax* was detected in 15 individuals, 8 from Equatorial Guinea (9.5% of infected individuals) and 7 from Angola (2.8% of infected individuals). From these 15 cases, 5 exhibited a single *P. vixax* infection, 8 a mixed infection with *P. falciparum* and 2 a triple infection with *P. falciparum* and *Plasmodium malariae*. In Equatorial Guinea, the overall prevalence of infected mosquitoes was 26.7% (219/819). From these, *P. vivax* infections were found in 10.9% (24/219), both in head/thorax (salivary glands) and abdomen (midgut): 22 were a single *P. vixax* infection and 2 a mixed infection with *P. falciparum*.

Genotyping of Pvcsp genes

Using the endonuclease AluI the fragments obtained for the *P. vivax* classic were: 243, 135, 133, 108, 90, 78, 57, 54, 30, 27 bp and for *P. vivax* VK247 were: 673, 243, 90, 78 bp. Using the endonuclease DpnI it was possible to identify fragments of 969, 71 and 50 bp in the case of *P. vivax* classic, and fragments of 360, 225, 108, 81, 71, 54, 50, 27 bp for *P. vivax* VK247. Fragments below 50 bp were not considered for variant determination due to the low molecular weight.

According to this, it was possible to identify 6 blood samples infected with *P. vivax* classic, 6 blood samples with *P. vivax* VK247 and 3 blood samples infected with two strains of *P. vivax*: classic and VK247.

No samples were identified as being infected with *P. vivax-like*. In this case, it was expected to obtain fragments of 786, 101, 83, 70 and 62 bp when using AluI, and fragments of 883, 169 and 50 bp when using DpnI.

For the 24 mosquitoes infected with *P. vivax*, the same procedure was used for the parasite characterisation but unfortunately no successful amplification of specific sequences was achieved.

Table 1. Prevalence of infection in both humans and mosquitoes, in Angola and Equatorial Guinea.

Prevalence of infection	Individuals		Mosquitoes
	Angola	Equatorial Guinea	
n	898	97	819
Overall infection	28.9% (245/848)	86.6% (84/97)	26.7% (219/819)
Overall infection F	97.9% (240/245)	95.2% (80/84)	89.0% (195/219)
Overall infection V	2.8% (7*/245)	9.5% (8*/84)	10.9% (24/219)
V	3 ind.	2 ind.	22 mosq.
F+V	3 ind.	5 ind.	2 mosq.
F+V+M	1 ind.	1 ind.	0

*All Duffy-negatives.
n - Sample size; F: *P. falciparum*; V: *P. vivax*; F+V: mixed infection by *P. falciparum* and *P. vivax*; F+V+M: mixed infection by *P. falciparum*, *P. vivax* and *P. malariae*.
doi:10.1371/journal.pntd.0001192.t001

Genotyping of Duffy blood group

All the human isolates *P. vivax* infected were genotyped for the Duffy gene by PCR-RFLP (82, 77 and 64 bp for Duffy positive genotypes and 82, 65, 64 and 12 bp for Duffy negative genotypes; the fragment of 12 bp was not considered due to the low molecular weight, not visible in gel). Results showed that all samples analysed were genotyped as FY*B-33/FY*B-33 (Duffy-negative homozygous) being therefore classified as Fy(a−b−).

Given that differentiation of bands in acrylamide gel is sometimes dubious, some samples were sequenced to confirm results. Sequencing (figure 2) confirmed the Duffy-negative genotype, since all of them contained the point mutation -33T→C.

Discussion

Despite all the efforts that have been made to control malaria, many of them having a real effect, the prevalence of infection is still very high, even in countries with active control campaigns, like Equatorial Guinea (86.6%) and Angola (28.9%).

Particularly, *P. vivax* seems not only to be evolving and adapting, causing more severe forms of the disease [6,8,34,35,36] but also appears to be more frequent in countries where either it was not present or it was not detected by the available techniques in the past, as is the case of some countries of West and Central Africa like, Congo [37], São Tomé and Principe [37,38], Gabon [37,39] and Cameroon [37], becoming a major source of concern. Our results corroborate these assumptions, since for the first time we were able to detect *P. vivax* on mainland Equatorial Guinea in humans and mosquitoes, which imply well-established whole life-cycles and active transmission.

Further, a relevant aspect needs to be stressed - the proportion of *P. vivax* infected mosquitoes is higher than the proportion of *P. vivax* infected individuals. This may be associated with the fact that in the human host this parasite may be "hidden" since it forms dormant forms in the liver – hypnozoites - and go unnoticed, being much more "visible" in mosquitoes. If this is the case, these results suggest that the prevalence of this species may be underestimated, not only in this country but in other parts of Africa.

Other factors associated with parasite-human interaction and immune response could be conditioning this variable prevalence in *P. vivax* infection in mosquitoes and human host.

In this study we were able to detect Duffy negative people carrying *P. vivax* infections, both in Angola and Equatorial Guinea, two countries located in West Africa, where the prevalence of Duffy negative individuals is near 95% [11], confirming thereby the suspicion of some authors [18,22–25]. Similar results were

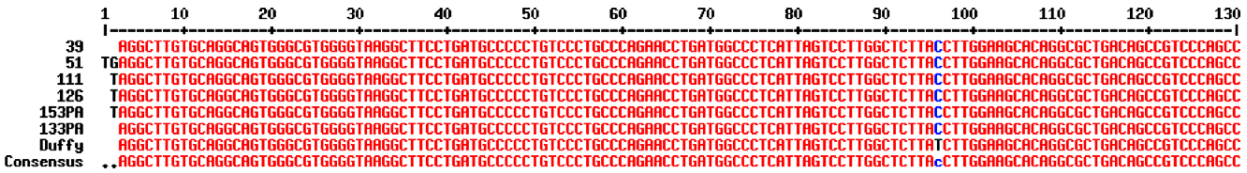

Figure 2. Multiple sequences alignment of promoter region from the DARC gene, allele FY*B, in the GATA box region.
doi:10.1371/journal.pntd.0001192.g002

found in other studies, but always in areas where the prevalence of Duffy positive is significantly higher: in Kenya - East Africa [22], Amazon region in Brazil [23,24] and more recently, in Madagascar [25].

Ménard *et al.* [25] suggested that Duffy positive individuals may serve as a reservoir of *P. vivax* providing an opportunity for this parasite to infect hepatocytes of Duffy negative people and the selection of new *P. vivax* strains with capacity to invade Duffy negative erythrocytes. In the present case, it is likely that the evolutionary process has been the same, although these two countries showed low prevalence of Duffy positive autochthonous individuals. From the beginning of the 90 s, these countries have experienced a marked increase in economic development with the finding of important oil reserves. Related to this development, intensive migration processes are occurring from outside and inside of the African continent. Therefore, workers from countries with higher Duffy positive and *P. vivax* prevalence could be circulating in Angola and Equatorial Guinea, thus increasing the reservoir of *P. vivax*.

Although we do not know which main force was contributing for the evolution of *P. vivax* and why it is able to infect Duffy negative erythrocytes, one thing seems to be clear - *P. vivax* may have an extraordinary ability to adapt. In addition, the African continent has both the ideal temperature and highly competent vectors for its transmission [3,40]. Altogether, these factors show that this parasite can become a serious public health problem in West and Central Africa, both for locals and travelers.

The results obtained in this work are highly relevant. First, it demonstrates that *P. vivax* is able to invade erythrocytes using other receptors than Duffy, and this new capacity is not exclusive of one strain of *P. vivax*, since we found samples infected with two different strains: VK247 and VK210/classic. Other species of *Plasmodium*, as *Plasmodium knowlesi* (phylogenetically close to *P. vivax*) and *P. falciparum* have more than one receptor for the invasion of erythrocytes [41]. Considering that these two phylogenetically distant species have evolved in order to recognize more than one receptor for erythrocyte invasion, it is expected that *P. vivax* is also evolving, becoming capable of using more than one path of invasion.

Second, this parasite seems to be expanding, and now it can be found in areas where it was not present in the past. Some approaches to determine the distribution limits of *P. vivax* have been carried out, although areas with high prevalence of Duffy negative were virtually considered free of this parasite [3]. So it is expectable that the real distribution of this parasite is greater than that found by these authors.

In conclusion, this study present the first cases of Duffy negative individuals infected with different strains of *P. vivax* (VK247 and classic) in two West African countries. This finding reinforces the idea that this parasite is rapidly evolving, being able to use other receptors than Duffy to invade the erythrocytes.

The presence of *P. vivax* infection both in blood samples and mosquitoes indicates that this parasite is well adapted. Further, the higher number of infected mosquitoes shows that this species is more "visible" in mosquitoes and may go unnoticed if blood samples are only analyzed.

It is therefore important to establish the real distribution of *P. vivax*, since new and more aggressive cases of infection by this parasite are reported every day, in countries where this parasite has not been noticed before having significant implications in the design of control measures and implementation of prophylactic and therapeutic regimens.

Supporting Information

Checklist S1 STROBE checklist
(DOC)

Acknowledgments

We thank all families who accepted to participate in this study. We also thank researchers and technicians from the National Malaria Control Program from the Republic of Equatorial Guinea's Ministry of Health and Social Welfare and "Centro de Referencia para el Control de Endemias" (Instituto de Salud Carlos III, Equatorial Guinea), and the Health Delegates of Health Care Units of Hospital, Programa Nacional de Controlo de Malária and Ministério da Saúde, Angola.

Author Contributions

Conceived and designed the experiments: APA. Performed the experiments: CM FD JF VGM. Analyzed the data: CM APA BdS VER AB PB. Contributed reagents/materials/analysis tools: APA PB VER AB. Wrote the paper: CM APA BdS JC. Biological material and data collection in the field: FD JC.

References

1. Guerra CA, Snow RW, Hay SI (2006) Mapping the global extent of malaria in 2005. Trends Parasitol 22: 353–358.
2. Galinski MR, Barnwell JW (2008) Plasmodium vivax: who cares? Malar J 7: S9.
3. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, et al. (2010) The International Limits and Population at Risk of Plasmodium vivax Transmission in 2009. PLoS Negl Trop Dis 4: e774.
4. Baird JK (2007) Neglect of Plasmodium vivax malaria. Trends Parasit 23: 533–539.
5. Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, et al. (2009) Key gaps in the knowledge of Plasmodium vivax, a neglected human malaria parasite. Lancet Infect Dis 9: 555–566.
6. Genton B, D'Acremont V, Rare L, Baea K, Reeder JC, et al. (2008) Plasmodium vivax and Mixed Infections Are Associated with Severe Malaria in Children: A Prospective Cohort Study from Papua New Guinea. PLoS Med 5: 881–889.
7. Rogerson SJ, Carter R (2008) Severe Vivax Malaria: Newly Recognised or Rediscovered? PLoS Med 5: e136.
8. Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, et al. (2008) Multidrug-Resistant Plasmodium vivax Associated with Severe and Fatal Malaria: A Prospective Study in Papua, Indonesia. PLoS Med 5: e128.
9. Alexandre MA, Ferreira CO, Siqueira AM, Magalhães BL, Mourão MPG, et al. (2010) Severe Plasmodium vivax Malaria, Brazilian Amazon. Emerg Infect Dis 16: 1611–1614.

10. Miller LH, Mason SJ, Dvorak JA, McGinniss MH, Rothman IK (1975) Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science 189: 561–563.
11. Langhi DM, Jr., Bordin JO (2006) Duffy blood group and malaria. Hematology 11: 389–398.
12. Culleton RL, Mita T, Ndounga M, Unger H, Cravo PVL, et al. (2008) Failure to detect Plasmodium vivax in West and Central Africa by PCR species typing. Malar J 7: 174.
13. Le Van Kim C (1995) Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 10: 224–228.
14. Castilho L, Rios M, Pellegrinno J, Jr., Saad STO, Costa FF, et al. (2004) A novel FY allele in Brazilians. Vox Sang 87: 190–195.
15. Carvalho TL, Ribolla PEM, Curi RA, Mota LS (2009) Characterization and transcriptional analysis of the promoter region of the Duffy blood group, chemokine receptor (DARC) in cattle. Vet Immunol Immunopathol 132: 153–159.
16. Cerávolo IP, Bruña-Romero O, Braga EM, Fontes CJ, Brito CF, et al. (2005) Anti-Plasmodium vivax duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. Am J Trop Med Hyg 72: 675–681.
17. Beeson JG, Crabb BS (2007) Towards a Vaccine against Plasmodium vivax Malaria. PLoS Med 4: 1862–1864.
18. Rosenberg R (2007) Plasmodium vivax in Africa: hidden in plain sight? Trends Parasitol 23: 193–196.
19. Rubio JM, Benito A, Berzosa PJ, Roche J, Puente S, et al. (1999) Usefulness of Seminested Multiplex PCR in Surveillance of Imported Malaria in Spain. J Clin Microbiol 37: 3260–3264.
20. Gautret P, Legros F, Koulmann P, Rodier MH, Jacquemin JL (2001) Imported Plasmodium vivax malaria in France: geographical origin and report of an atypical case acquired in Central or Western Africa. Acta Trop 78: 177–181.
21. Rubio JM, Benito A, Roche J, Berzosa PJ, García ML, et al. (1999) Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of Plasmodium vivax infection in Equatorial Guinea. Am J Trop Med Hyg 1999 Feb;60(2): 183–7.
22. Ryan JR, Stoute JA, Amon J, Dunton RF, Mtalib R, et al. (2006) Evidence for transmission of Plasmodium vivax among a duffy antigen negative population in Western Kenya. Am J Trop Med Hyg 75: 575–581.
23. Cavasini CE, de Mattos LC, Couto AA, Couto VS, Gollino Y, et al. (2007b) Duffy blood group gene polymorphisms among malaria vivax patients in four areas of the Brazilian Amazon region. Malar J 6: 167–175.
24. Cavasini CE, Mattos LC, Couto AA, Bonini-Domingos CR, Valencia SH, et al. (2007a) Plasmodium vivax infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? Trans R Soc Trop Med Hyg 101: 1042–1044.
25. Ménard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, et al. (2010) Plasmodium vivax clinical malaria is commonly observed in Duffy-negative Malagasy people. Proc Natl Acad Sci U S A 107: 5967–5971.
26. Qari SH, Shi YP, Goldman IF, Udhayakumar V, Alpers MP, et al. (1993) Identification of Plasmodium vivax-like human malaria parasite. Lancet 341: 780–783.
27. Kappe SH, Buscaglia CA, Nussenzweig V (2004) Plasmodium sporozoite molecular cell biology. Annu Rev Cell Dev Biol 20: 29–59.
28. Rosenberg R, Wirtz RA, Lanar DE, Sattabongkot J, Hall T, et al. (1989) Circumsporozoite protein heterogeneity in the human malaria parasite Plasmodium vivax. Science 245: 973–976.
29. Souza-Neiras WC, Storti-Melo LM, Cassiano GC, Couto VS, Couto AA, et al. (2010) Plasmodium vivax circumsporozoite genotypes: a limited variation or new subspecies with major biological consequences? Malar J 9: 178.
30. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE (1995) Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg 52: 565–568.
31. Arez AP, Lopes D, Pinto J, Franco AS, Snounou G, et al. (2000) – Plasmodium sp.: Optimal protocols for PCR amplification from mosquito (Anopheles sp.) samples. Exp Parasitol 94: 269–272.
32. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN (1993) Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of high prevalence of mixed infections. Mol Biochem Parasitol 58: 283–292.
33. Alves RT, Póvoa MM, Goldman IF, Cvasini CE, Rossit ARB, et al. (2007) A new polymerase chain/restriction fragment length polymorphism protocol for Plasmodium vivax circumsporozoite protein genotype (VK210, VK247 and P. vivax–like) determination. 59: 415–419.
34. Barcus MJ, Basri H, Picarima H, Manyakori C, Sekartuti, et al. (2007) Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua. Am J Trop Med Hyg 77: 984–991.
35. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, et al. (2007) Vivax malaria: neglected and not benign. Am J Trop Med Hyg 77: 79–87.
36. Kochar DK, Das A, Kochar SK, Saxena V, Sirohi P, et al. (2009) Severe Plasmodium vivax malaria: a report on serial cases from Bikaner in northwestern India. Am J Trop Med Hyg 80: 194–198.
37. Gautret P, Legros F, Koulmann P, Rodier MH, Jacquemin JL (2001) Imported Plasmodium vivax malaria in France: geographical origin and report of an atypical case acquired in Central or Western Africa. Acta Trop 78: 177–181.
38. Snounou G, Pinheiro L, Antunes AM, Ferreira C, do Rosario VE (1998) Nonimmune patients in the Democratic Republic of Sao Tome e Principe reveal a high level of transmission of P. ovale and P. vivax despite low frequency in immune patients. Acta Trop 70: 197–203.
39. Poirriez J, Landau I, Verhaeghe A, Savage A, Dei-Cas E (1991) [Atypical forms of Plasmodium vivax. Apropos of a case]. Ann Parasitol Hum Comp 66: 149–154.
40. Collins WE, Roberts JM (1991) Anopheles gambiae as a host for geographic isolates of Plasmodium vivax. 7: 569–573.
41. Michon P, Stevens JR, Kaneko O, Adams JH (2002) Evolutionary relationships of conserved cysteine-rich motifs in adhesive molecules of malaria parasites. Mol Biol Evol 19: 1128–42.

Chapter 4 - Molecular evidence of positive selection in the transglutaminases genes in *Anopheles gambiae*

This chapter is a paper in preparation:

Mendes C., Custódio A., Pinto J., Arez AP., Silveira H. Molecular evidence of positive selection in transglutaminases of *Anopheles gambiae*.

Molecular evidence of positive selection in transglutaminases of *Anopheles gambiae*

Cristina Mendes, Ana Custódio, João Pinto, Ana Paula Arez, Henrique Silveira[§]

Centro de Malária e outras Doenças Tropicais, Unidade de Parasitologia Médica, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal

[§]Corresponding author

Keywords

Anopheles gambiae, transglutaminase 1, transglutaminase 2, positive selection

Abstract

Several defence mechanisms in the mosquito vector *Anopheles gambiae* control malaria infection by limiting the number of parasites. These defence mechanisms include a wide variety of cellular and humoral responses performed by various organs and cell types. Transglutaminases (TG) are a class of proteins involved in coagulation and wound healing. *Anopheles gambiae* have three TG: AgTG1, AgTG2 and AgTG3. The last one is a male exclusive TG associated to sperm coagulation and AgTG2 silencing increases mosquito infection by *Plasmodium*, but little is known about the functions of AgTG1 and AgTG2 and their role upon parasite invasion.

Genetic diversity of *A. gambiae AgTG1* and *AgTG2* was investigated in samples collected in Guinea Bissau on individual households and classified as infected and non-infected mosquitoes. The two genes studied are highly polymorphic, especially *AgTG2*. No significant genetic differentiation for the genes studied was found either between *A. gambiae* molecular forms, or between infected and non-infected groups.

The Ka/Ks ratio (nonsynonymous substitutions/synonymous substitutions) was higher than one for *AgTG2*, which is consistent with positive selection. For *AgTG1*, a Ka/Ks<1 was consistent with purifying selection. When a codon-by-codon approach was used, some codons (17 and 11 in *AgTG1* and 50 and 41 in *AgTG2,* for the comparisons between infected/non-infected mosquitoes and for M- and S-forms) displayed Ka/Ks>1 suggesting that positive selection is acting in some specific regions of the gene. Most sites under positive selection were found to fall in the coding region of both genes, and the majority of the substitutions were located in the periphery, as seen in the 3D model of the protein.

Results suggest that these genes may play a role in pathogen recognition, being involved in the response against the infection.

Introduction

It is known that host-pathogen interactions are a powerful driving force of adaptive evolution, which can lead to changes in the genes involved in the process (Haldane, 1949). Molecular evolutionary studies showed that this evolutionary dynamics leaves traces in the genomes of both hosts, being immunity related genes among those with a higher rate of adaptive evolution (Clark et al., 2003; Schlenke & Begun 2003; Nielsen et al., 2005; Tiffin & Moeller 2006; Sackton et al., 2007). *Plasmodium* can exert the same kind of selective pressure not only in the human host - as previously demonstrated by alteration in genes such as glucose 6-phosphate dehydrogenase gene, thalassemia α+, haemoglobin S and the Duffy blood group (review in López et al., 2010; Hedrick, 2012) – but also in the mosquito vector. Several studies demonstrated that innate immune related genes have been targets of positive

selection, which may play a role in protection against *Plasmodium* infection (Christophides et al., 2002, Crawford et al., 2012).

There are evidences that *Anopheles gambiae*, the major malaria vector in Sub-Saharan Africa, is capable of trigger a response against *Plasmodium falciparum* limiting the infection by reducing the number of parasites (Blandin & Levashina, 2004; Whitten et al., 2006; Barrilas-Mury, 2007). These defence mechanisms involve not only structural barriers but also a strong innate immune response against the parasite, including a wide variety of cellular and humoral mechanisms performed by various organs and cell types. Transglutaminases (TG) are a class of proteins involved in coagulation and wound healing. While most invertebrates possess a maximum of two, anopheline mosquitoes have three distinct TGs (Rogers et al., 2009): AGAP009100 or *AgTG1*; AGAP009098 or *AgTG2* and AGAP009099 or *AgTG3* (Le, 2013). *AgTG3*, is a male exclusive TG associated to sperm coagulation (Rogers at al., 2009, Le et al., 2013), which excludes this specific TG from being involved in malaria transmission. Previous studies associated *AgTG2* to an increased resistance against *Plasmodium* infection, after *A. gambiae* immune system stimulation (Silveira et al., 2012). However, little is known about *AgTG1* and *AgTG2* functions in the mosquito and upon parasite invasion.

In this study, through a well characterized sample of field-collected infected and non-infected *A. gambiae* mosquitoes, we analysed patterns of molecular evolution of two TG genes - *AgTG1* and *AgTG2*, in order to investigate if interactions between mosquito and parasite may have exerted selective pressure upon these genes.

Methods

Mosquito sampling

Anopheles gambiae mosquitoes were collected both in Guinea Bissau and Equatorial Guinea. Details of the study area and collection procedures of mosquito specimens collected in Guinea Bissau were previously described in Arez et al., (1997 and 2003). Briefly, blood-fed resting *A. gambiae* s.l. females were collected inside bed nets in each household surveyed and kept in paper cups for 8 days with glucose *ad*

libitum, allowing the development of *Plasmodium* species. After this period, mosquitoes were dissected. Midguts and salivary glands were collected in tubes with 25ml of lysis buffer supplemented with proteinase K and kept a -20ºC until DNA extraction, used for *Plasmodium* species identification. The remainder of the carcasses were kept dry in silica gel filled individual tubes for mosquito species identification.

In Equatorial Guinea, the mosquito specimens were collected using the same methodology described above, with slightly modifications as described in Mendes et al., 2011 and 2013. Shortly, individual mosquitoes, dried on silica gel, were stored at room temperature until DNA preparation.

DNA extraction and species identification

Two different methods were used for the DNA preparation: for the mosquito specimens from Guinea-Bissau, the DNA was obtained by phenol/phenol-chloroform extraction and ethanol precipitation, whereas for the Equatorial Guinea samples, the DNA was extracted using chelex protocol described by Arez et al. (2000).

The *A. gambiae* complex comprises at least 7 sibling species and within the nominal species, two molecular forms, denoted M and S, have been described (Coetzee et al., 2013 and refs there in). The M-form was recently named *Anopheles coluzzii* while the S form retained the nominotypical name *A. gambiae* (Coetzee et al., 2013). For simplification, the terms M and S will be used throughout the text when referring to *A. coluzzii* and *A. gambiae*, respectively. Species identification of the members of the *A. gambiae* complex was carried out by PCR-RFLP as described in Fanello et al., (2002).

Parasite species identification was performed by nested-PCR (Snounou et al., 1993). Mosquitoes were classified as infected or non-infected if presence of *P. falciparum* DNA was detected by PCR. As mosquitoes were collected inside the bed net, in which the infected individual was sleeping and were all engorged, we assumed that those mosquitoes infected at day 8 post-blood meal were more susceptible to infection.

Transglutaminase sequencing

Primers were designed based on the TG sequences from *A. gambiae* genome at Vector Base (AGAP009098; AGAP009100). Name, sequence of each pair of primers PCR condition and product length are summarized in Table S1 [Table S1, suppl. Mat]. Nested PCR reactions were performed in a MyCycler™ Thermal cycler (Biorad) with final reagent concentrations of 1× PCR reaction buffer (Promega), 1.5 mM of $MgCl_2$, 2μM dNTP's, 0.3 μM of each primer and 1.25 U/μl of Taq DNA Polymerase (Promega) for all reactions.

Amplified products were visualized under UV light in 2% ethidium bromide stained agarose gels after electrophoresis and products presenting the expected length were purified using SureClean Kit (Bioline) according to manufacturer's recommendations. After purification, all samples were sequenced in both directions at Macrogen Inc. laboratories (The Netherlands).

Data analysis

All sequences were aligned using BioEdit Sequence Alignment Editor version 7.1.3.0 (Hall, 1999). DnaSP version 5.10 (Librado & Rozas, 2009) was used to determine, for each group (*i.e.* infected/non-infected and M-form/S-form) the number of segregating sites (S) and nucleotide pairwise diversity (π). The genetic diversity (h) (Nei 1987) was determined using the Arlequin software version 3.11 (Excoffier et al., 2005).

To test deviations from neutrality, four tests were performed: Tajima's D test (1989); Fu & Li's D* and F* tests (1993) and the Ka/Ks ratio. Tajima's D test compares the total number of mutations observed (η) and the average number of nucleotide differences between two sequences (π_n), whereas Fu & Li's D* and F* test compares the numbers of mutations in internal branches (η_i) and the numbers of mutations in external branches of the gene genealogy (η_e). Under neutrality, the estimators should be equal, so that Fu & Li's D* and F* values should be approximately zero for a gene under neutrality. Finally, Ka/Ks ratio compares the number of non-synonymous (Ka) substitutions and the number of synonymous (Ks) substitutions. Under neutrality, Ka/Ks

ratio should be equal to one; Ka/Ks ratios higher than one indicate positive selection, whereas ratios smaller than one indicate purifying selection.

In addition, the Ka/Ks ratio was applied to the coding region of both genes using a codon by codon maximum likelihood approach implemented in DNAsp. This type of analysis would allow the identification within each gene, the regions under neutrality and the ones subjected to different types of selection.

The sequence-based *F* statistic (*Fst*) calculated according to Hudson et al. (1992) was used as a pairwise genetic distance measure between groups. Statistical significance of Fst values was assessed by 1000 bootstrap replications. In order to estimate the total percentage of variance attributable to differences between molecular forms (M and S) and among infected and non-infect groups, an analysis of molecular variance (AMOVA) was performed (Excoffier et al., 1992). These analyses were performed in the Arlequin software version 3.11 (Excoffier et al., 2005).

Protein diversity

Amino acid sequences were obtained using the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/) and aligned with ClustalW program (Larkin et al., 2007). Each protein sequence was modelled using Swissmodel at http://swissmodel.expasy.org/workspace/index.php. Nonsynonymous mutations were visualized in each model using the Swiss-PdB viewer v. 4.0.1. (Arnold et al., 2006; Schwede et al., 2003; Guex & Peitsch, 1997), in order to identify possible structural alterations. Phylogenetic analysis was performed with the MEGA 5.1 software (Kumar et al., 2008), using a distance-based Neighbour-Joining, following the Jukes-Cantor method (Jukes & Cantor, 1969). Bootstrap values were calculated from 1000 replicates (Kumar et al., 2008).

Results

For the mosquito specimens collected in Equatorial Guinea was only possible to obtain four sequences: all from *AgTG2* gene. Due to the unsuccessful amplification of

these two genes; probably due to DNA degradation; a small number of sequences were obtained and therefore samples from Equatorial Guinea were removed from all analyses, as it was not possible to make comparisons with such a small number of samples.

For Guinea-Bissau, a total of 46 TG sequences were obtained from 34 *A. gambiae* mosquitoes: 22 *AgTG1* sequences from 13 infected (7 M-form and 6 S-form) and 9 non-infected mosquitoes (6 M-form and 3 S-form) and 24 *AgTG2* sequences from 13 infected (9 M-form and 4 S-form) and 11 non-infected mosquitoes (8 M-form and 3 S-form).

Polymorphism and diversity

For the *AgTG1* gene, a sequence of 3270 bp, which contains the entire coding region (2205bp), was amplified. For the *AgTG2* gene, a sequence of 3143bp was obtained, but it did not include the entire coding region (only 2355bp of the 2515bp).

For *AgTG1*, the nucleotide diversity in the coding region (0.007 – 0.023) was always lower than the one found for non-coding regions (0.008 – 0.087). Similar results were obtained for the *AgTG2* gene, where the nucleotide diversity (0.007 – 0.033) found in the coding region was lower than in non-coding region (0.005 – 0.046) except in exons 1 and 2 (Table 1). The analysis of nucleotide diversity in the non-synonymous sites, revealed that these two genes are remarkably polymorphic, especially *AgTG2*.

Both *A. gambiae* molecular forms presented similar values concerning the overall nucleotide diversity, with the M-form presenting lower values of π when compared to the S-form. However, when comparing infected and non-infected mosquitoes, the first group showed higher nucleotide diversity in both genes.

AgTG1 and AgTG2 genes presented lower number of synonymous substitutions than non-synonymous substitutions (Table 1). Total number of polymorphic sites was similar between the two genes (141 for *AgTG1* and 137 for *AgTG2*). Infected mosquitoes showed a lower number of polymorphic sites in both genes (*AgTG1*: 114 *versus* 121; *AgTG2*: 125 *versus* 129; infected *versus* non-infected respectively). The M-form presented a lower number of polymorphic sites for *AgTG1*, when compared with the S-

Table 1 – Intraspecific polymorphism for *A. gambiae AgTG1* and *AgTG2* genes.

Genes			***AgTG1***					***AgTG2***				
			Ag_M-form	**Ag_S-form**	**I**	**NI**	**Total**	**Ag_M-form**	**Ag_S-form**	**I**	**NI**	**Total**
	N		13	9	13	9	22	17	7	13	11	24
5'	**L**		**599**					**564**				
	S		17	18	21	13	21	13	9	10	12	14
	Π		0.009	0.011	0.012	0.008	0.010	0.007	0.006	0.005	0.008	0.007
Exon 1	**L**		**523**					**107**				
	S	11	12	13	11	14	1	3	0	3	1	
		1	0	1	0	1	5	8	5	9	7	
	Π		0.008	0.007	0.007	0.008	0.007	0.030	0.033	0.024	0.040	0.028
Intron	**L**		**72**					**80**				
	S		12	9	9	12	14	2	2	2	2	2
	Π		0.069	0.052	0.036	0.087	0.064	0.013	0.007	0.010	0.014	0.012
Exon 2	**L**		**384**					**555**				
	S	1	6	1	6	6	5	4	4	4	5	
		12	26	11	25	28	28	26	29	28	29	
	Π		0.012	0.023	0.010	0.025	0.017	0.022	0.022	0.020	0.023	0.021
Intron	**L**		**63**					**72**				
	S		2	7	7	5	7	7	5	7	6	7

	Π	0.010	0.035	0.025	0.022	0.022	0.032	0.034	0.038	0.028	0.033
Exon 3	**L**	**650**					**1493**				
	S 1	4	3	4	4	9	8	8	7	9	
	16	16	17	17	18	46	40	45	49	49	
	Π	0.007	0.012	0.008	0.012	0.009	0.013	0.014	0.013	0.014	0.013
Intron	**L**	**57**					**73**				
	S	2	2	2	2	2	7	6	7	7	7
	Π	0.012	0.008	0.010	0.011	0.010	0.041	0.045	0.042	0.046	0.042
Exon 4	**L**	**435**					**200**				
	S 8	7	8	7	8	8	5	7	5	8	
	1	0	1	1	1	0	0	0	0	0	
	Π	0.009	0.008	0.009	0.008	0.008	0.012	0.008	0.012	0.007	0.010
Intron	**L**	**68**					**-**				
	S	2	3	3	3	3	-	-	-	-	-
	Π	0.012	0.025	0.021	0.015	0.018	-	-	-	-	-
Exon 5	**L**	**213**					**-**				
	S 8	7	8	8	8	-	-	-	-	-	
	1	1	1	1	1	-	-	-	-	-	
	Π	0.017	0.015	0.016	0.018	0.017	-	-	-	-	-
3'	**L**	**206**					**-**				

	S	10	6	10	7	9	-	-	-	-	-
	Π	0.018	0.013	0.017	0.012	0.014	-	-	-	-	-
Total	**L**	**3270**					**3143**				
	S	104	122	114	121	141	132	113	125	129	137
	Π	0.011	0.014	0.011	0.014	0.012	0.015	0.015	0.014	0.016	0.015
	Πs	0.020	0.024	0.019	0.025	0.022	0.009	0.010	0.008	0.010	0.009
	Πns	0.006	0.009	0.006	0.010	0.007	0.018	0.018	0.018	0.019	0.018
	h	0.012	0.014	0.012	0.014	0.012	0.016	0.016	0.015	0.016	0.015

Ag_M-form – *A. gambiae* M-form; Ag_S-form – *A. gambiae* S-form; I – Infected mosquitoes; NI – Non-infected mosquitoes; L, Length; S, segregating sites n- total number of nonsynonymous changes; s – total number of synonymous changes; Π, nucleotide diversity; Πs, average pairwise nucleotide diversity at synonymous sites; Πns, average pairwise nucleotide diversity at non-synonymous sites; *h*, genetic diversity.

form (104 *versus* 122), whereas for *AgTG2*, the M-form showed a higher number of polymorphic sites (132 *versus* 113).

Genetic diversity (*h*) estimates were similar either between forms (*AgTG1*: 0.012 *versus* 0.014; *AgTG2*: 0.016 *versus* 0.016; M-form *versus* S-form respectively) or infected and non-infected mosquitoes (*AgTG1*: 0.012 *versus* 0.014; *AgTG2*: 0.015 *versus* 0.016; infected *versus* non-infected respectively) (Table 1).

Population differentiation and Phylogeny

The *Fst* value was calculated for each gene of *A. gambiae* M- and S-form. *Fst* values ranged from 0 to 0.007 in *AgTG1* gene and from 0 to 0.004 for *AgTG2* gene, and all comparisons were non-significant [Table S2, suppl. Mat].

Through the analyses of the phylogenetic trees (Figure 1A and B), no clear separation was observed neither between the two forms of *A. gambiae* nor between infected and non-infected groups.

Infected (with M- and S-forms) and non-infected group (with M- and S-forms) were compared through an AMOVA and no significant percentage of variation was found, either between the two groups analysed or within each group, for both genes (Table S3 and S4, suppl. Mat).

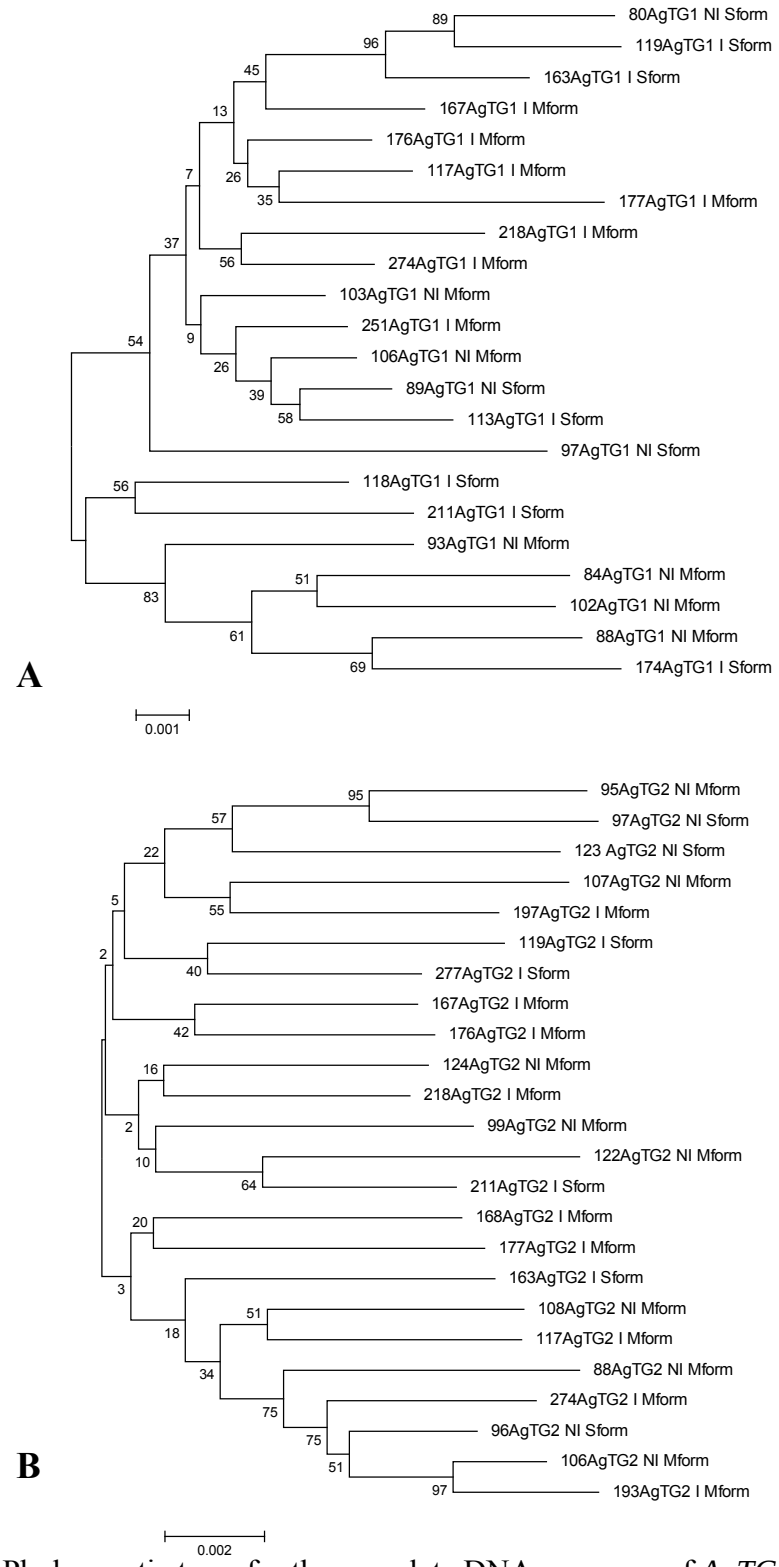

Figure 1: Phylogenetic trees for the complete DNA sequence of *AgTG1* (**A)** and *AgTG2* (**B)** genes.

NI – non-infected mosquitoes; I – infected mosquitoes; Sform - *A. gambiae*; S-form; Mform - *A. gambiae* M-form

Selection tests

To test if the two genes were under the influence of selection and if *Plasmodium* may be acting as a driving force to TG genes evolution, firstly we analysed infected and non-infected mosquitoes regardless the molecular form, given that there was no significant inter-form differentiation in both *AgTG1* and *AgTG2* genes, and then M- and S-forms regardless the infection *status* of the mosquitoes.

For the comparison between infected and non-infected mosquitoes, overall, Tajima's D and Fu & Li D* and F* tests were non-significant suggesting no departures from neutrality in both genes (Table 2). However, when the totally of the *AgTG2* gene was analysed, the Fu & Li's D* and F* test gave a significant value above 0, which is indicative of balancing selection, but this may also be associated with population expansion (Barreiro & Quintana-Murci, 2010).

To overcome this doubt, genes were also screened for non-synonymous/synonymous substitution ratios (Ka/Ks), a test that is not sensitive to demographic events. Using this test evidence for positive selection was found for *AgTG2* gene, whose ratio is higher than one but not for *AgTG1* for which a Ka/Ks ratio lower than one suggests purifying selection (Table 2).

When Ka/Ks ratios were calculated using a codon-by-codon approach, there were 17 codons with significant Ka/Ks>1 in *AgTG1* and 50 codons with Ka/Ks>1 in *AgTG2*, suggesting that particular regions at each gene may be under the effect of positive selection (Figure 2A and B).

When the comparisons were made between M- and S-forms, once again the Tajima's D and Fu & Li D* and F* tests were non-significant suggesting no departures from neutrality in both genes (Table 2). However it is important to highlight that in both genes, the M-form presented positive values of Tajima's D and Fu & Li D* and F*, which is indicative of balancing selection and the S-form always presented negative values of Tajima's D and Fu & Li D* and F*, which is indicative of positive selection.

The analysis of the ratio Ka/Ks showed evidences for positive selection for *AgTG2* gene but not for *AgTG1* (Table 2).

Table 2: Neutrality tests for the two transglutaminases genes *AgTG1* and *AgTG2* in Infected mosquitoes, Non-Infected mosquitoes, *A. gambiae* M-form and *A. gambiae* S-form.

Genes	Groups	Fu & Li's		Tajima's D	Ka/Ks
		D*	F*		
AgTG1	**Infected**	-0.14 ^{NS}	0.25 ^{NS}	-0.41 ^{NS}	0.333
	Non-Infected	-0.04 ^{NS}	-0.01 ^{NS}	0.08 ^{NS}	
	***A. gambiae* M-form**	0.13^{NS}	0.09 ^{NS}	-0.06 ^{NS}	0.312
	***A. gambiae* S-form**	-0.01^{NS}	-0.04^{NS}	-0.10 ^{NS}	
	Total	-0.001 ^{NS}	-0.08 ^{NS}	-0.22 ^{NS}	-
AgTG2	**Infected**	0.46^{NS}	0.47 ^{NS}	0.28 ^{NS}	1.402
	Non-Infected	0.77^{NS}	0.81 ^{NS}	0.52 ^{NS}	
	***A. gambiae* M-form**	1.13^{NS}	1.20^{NS}	0.78^{NS}	2.370
	***A. gambiae* S-form**	-0.02^{NS}	-0.04^{NS}	-0.10^{NS}	
	Total	1.44*	1.48**	0.86 ^{NS}	-

^{NS} – non significant; * P‹0.05; ** 0.10 ‹ P ‹ 0.05.

When Ka/Ks ratios were calculated using a codon-by-codon approach, there were 11 codons with significant Ka/Ks>1 in *AgTG1* and 41 codons with Ka/Ks>1 in *AgTG2*, suggesting that particular regions at each gene may be under the effect of positive selection (Figure 2C and D).

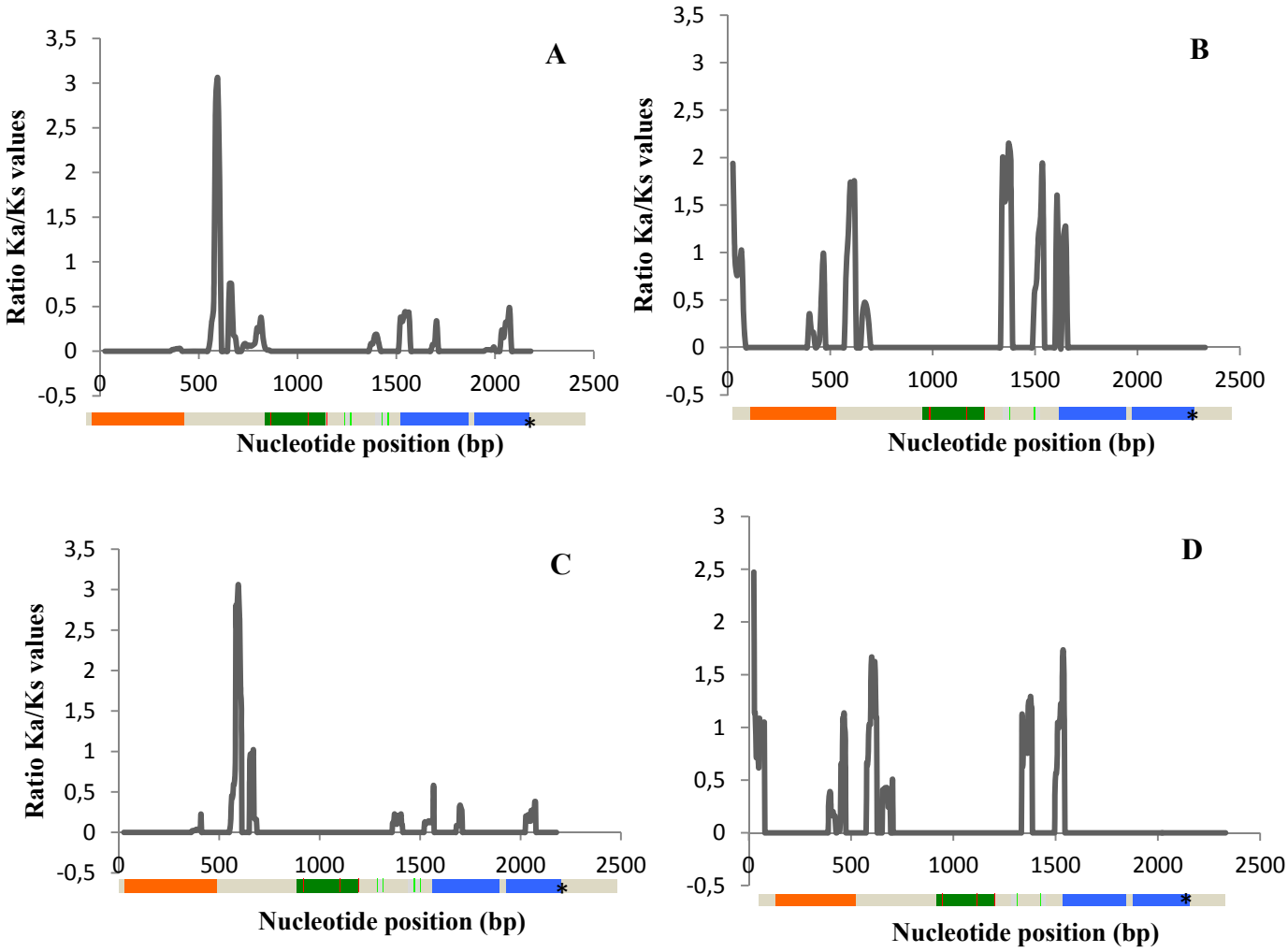

Figure 2: Values of Ka/Ks ratio for the coding region of:

A- *AgTG1* gene – comparison between infected and non-infected mosquitoes
B- *AgTG2* gene – comparison between infected and non-infected mosquitoes
C- *AgTG1* gene – comparison between M- and S- forms
D- *AgTG2* gene – comparison between M- and S- forms

The nucleotide sequence is numbered from the first base position at the 5' end. Amino acids are numbered from the initiating start codon methionine (M). The transglutaminase signatures N- and C- terminal and core are within orange (V22-L156 for *AgTG1* gene and E34-E176 for *AgTG2* gene), blue (R521-Q620, P628-P725 for *AgTG1* gene and V541-Q647, P655-E752 for *AgTG2* gene) and green (P300-395 for *AgTG1* gene and P319-P411 for *AgTG2* gene) boxes, respectively. The catalytic triads are shown in red (C306, H365 and D392 for *AgTG1* gene and C325, H384 and D408 for *AgTG2* gene). Amino acids residues potentially involved in calcium binding are shown in light green (N432, H434, E482 and E487 for *AgTG1* gene and N450, D450 and E498 for *AgTG2*gene). The termination codon is indicated by the symbol *

Protein analysis

For AgTG1 protein, 12 different variants were found in 22 sequences analysed. The two most common variants (AgTG1-H1 and AgTG1-H2) were shared between the infected and non-infected groups, whereas AgTG1-H3 variant was only present in the non-infected group (Figure 3).

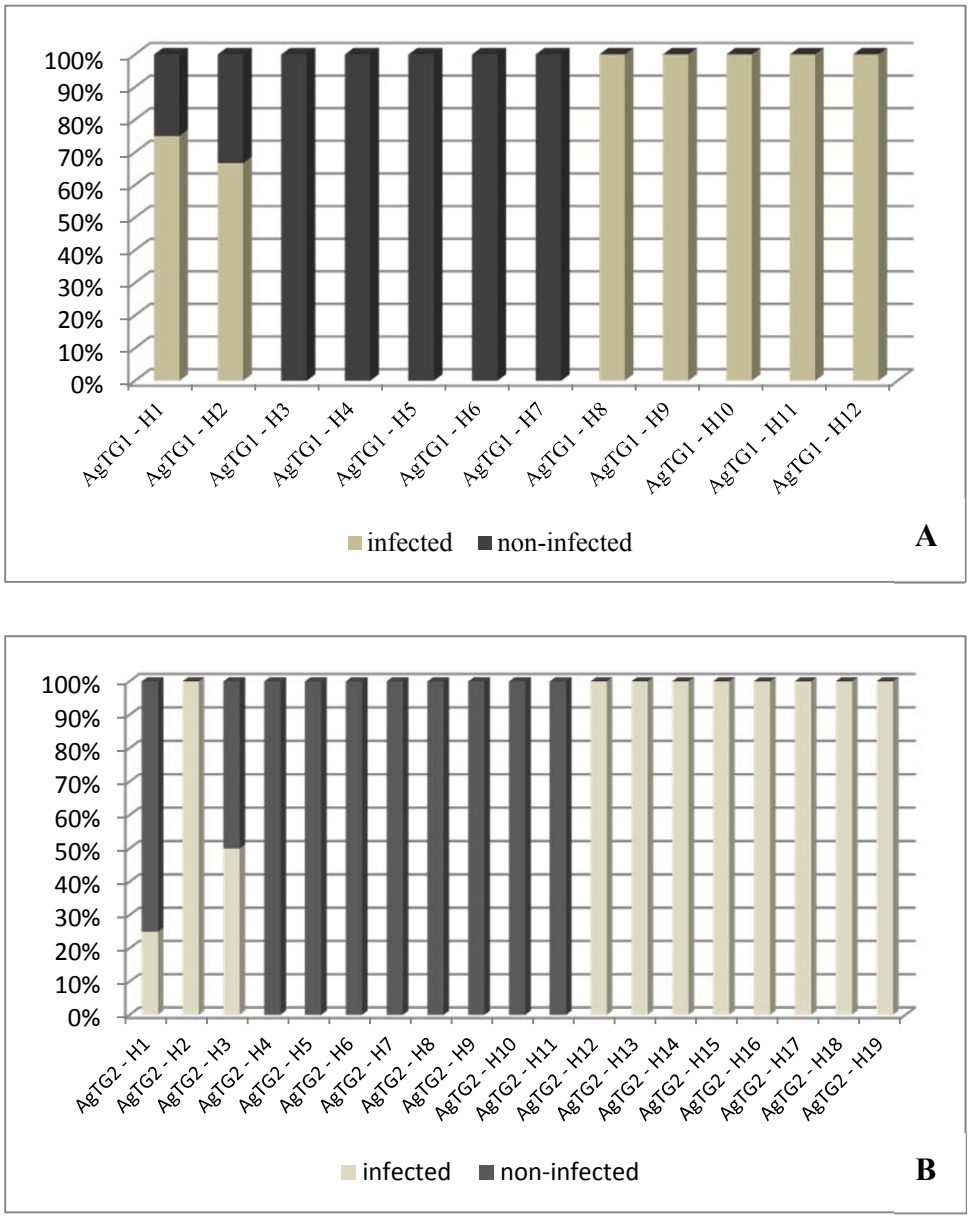

Figure 3: Distribution of the different variants of the AgTG1 (**A**) and AgTG2 (**B**) proteins in the non-infected and infected mosquitoes.

Higher diversity was observed for AgTG2, with the majority of sequences analysed corresponding to unique variants: 19 different variants in 24 samples analysed. Three variants occurred in more than one mosquito sample: AgTG2-H1 is predominantly present in non-infected mosquitoes; AgTG2-H2 is present only in infected mosquitoes and AgTG2-H3 is shared by the two groups (Figure 3).

The best fitting 3D model for the AgTG1 protein was based on recombinant human cellular coagulation factor XIII (1f13A) and for AgTG2 was based on the crystal structure of the red sea bream TG (1g0dA).

Non-synonymous substitutions under selection (Ka/Ks ratio) appear not to be homogeneously distributed along the protein domains. In both proteins, the amino acid substitutions were mainly present at the periphery. No substitutions were found neither in the catalytic centre nor in the binding calcium domains (Figures 4 A and B).

Most of the substitutions in AgTG1 are distributed equally among groups but the substitution of a tyrosine for a phenylalanine at position 684 is more frequent in the infected group.

Regarding AgTG2, amino acid substitutions do not coincide in the infected and non-infected group, each occurring predominantly or in one group or another. For example, the asparagine in the position 493 (near a calcium binding domain), is only present in the non-infected group and the valine in the position 559 is only present in the infected group.

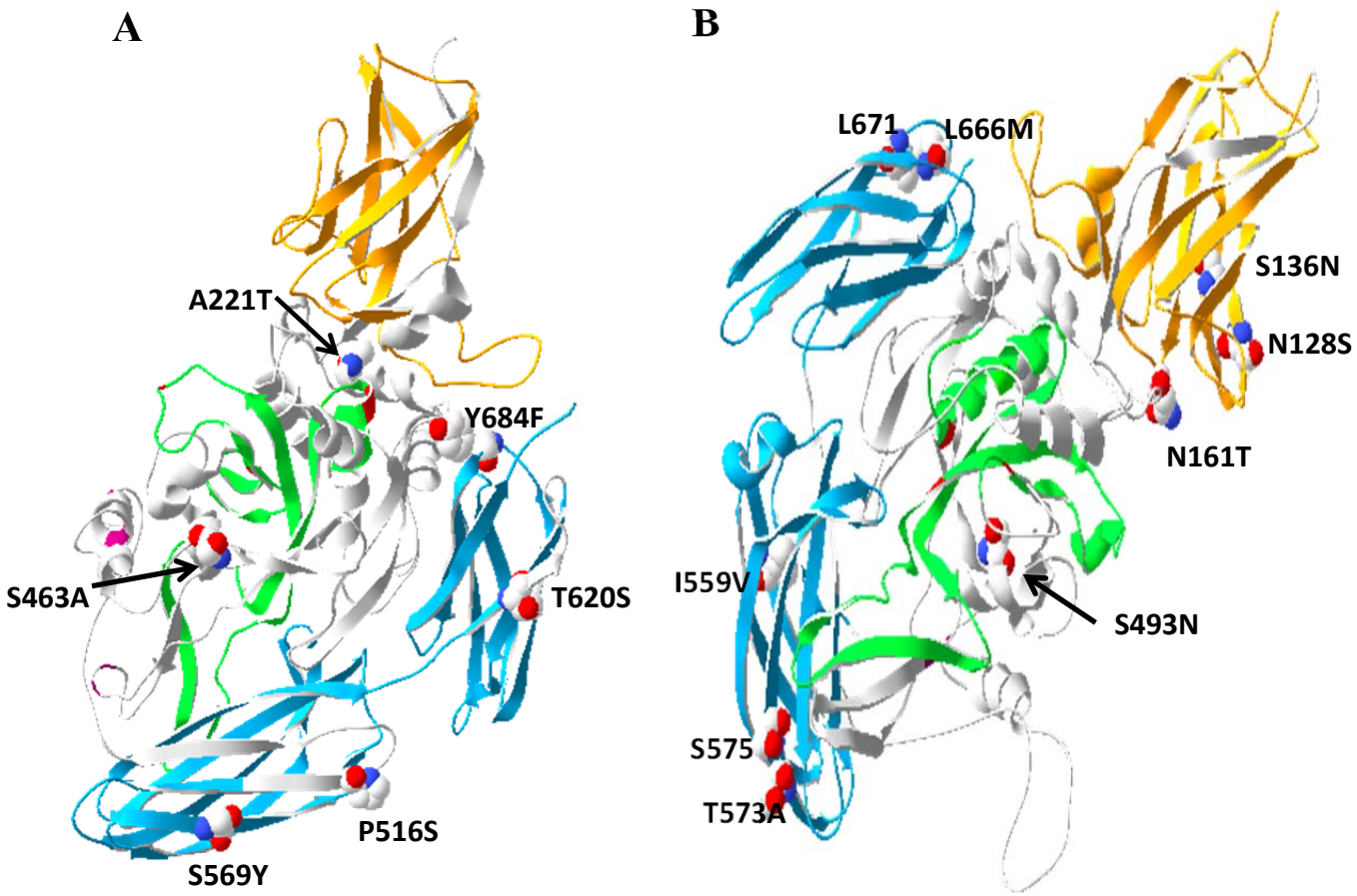

Figure 4: Structural model of AgTG1 protein (A) and AgTG2 protein (B). Three-dimensional (3D) structural localization of mutated amino acids represented in solid structures in blue, white and red.

The transglutaminase signatures N- and C- terminal and core are within orange, blue and green, respectively.

Discussion

The estimates of nucleotide diversity for the two genes analysed are in agreement with other studies that analysed immune related genes in *A. gambiae* (Morlais et al., 2004; Simard et al., 2007; Cohuet et al., 2008; Mendes et al., 2010) as well as in *Drosophila melanogaster* (Labate et al., 1999; Jiggins & Hurst, 2003).

When the levels of nucleotide diversity in the nonsynonymous sites were analysed, *AgTG2* gene is highly polymorphic when compared with other *A. gambiae* genes, including those related to immune function (Obbard et al., 2007; Simard et al., 2007; Slotman et al., 2007; Parmakelis et al., 2008; Lehman et al., 2009; Obbard et al., 2009). These values exceed the *A. gambiae* genome average diversity by 10-fold but are in concordance with data obtained for other genes evolving under positive selection, like TEP1 and APL1, revealing evidence of host-pathogen co-evolutionary dynamics (Obbard et al., 2008; Rottschaefer et al., 2011). Although the AgTGs are not immunity proteins, they are involved in coagulation and wound healing, mechanisms associated to immune responses (Wang et al., 2010; Shibata et al., 2013). Given this association between *AgTG1* and *AgTG2* and the immune system of *A. gambiae*, it is not surprising that as in other organisms, such as *Drosophila,* primates and plants, immune related genes tend to evolve adaptively and more rapidly than other genes in the genome (Clark et al. 2003; Schlenke & Begun 2003; Nielsen et al. 2005; Tiffin & Moeller 2006; Sackton et al. 2007; Obbard et al., 2008; Rottschaefer et al., 2011; Crawford et al., 2012). Higher diversity in immune-related genes can arise and be maintained over evolutionary time as a consequence of natural selective pressures to combat against a wide variety of pathogens.

Comparing the two molecular forms of *A. gambiae*, the values of nucleotide diversity found for the M-form of *A. gambiae* were lower than those found for the S-form, a fact, already observed for other genes (Cohuet et al., 2008). Even so, we found no significant differences using *Fst* estimates and the phylogenetic analysis, showing that the M- and S- forms do not present significant genetic differentiation in this sampling location. The degree of isolation between M- and S- forms, is not homogenous throughout the species distribution range. While Crawford et al. (2012) and Wondji et al. (2002) showed that M- and S- forms presented differences in haplotypic and nucleotide diversity, suggesting that molecular forms of *A. gambiae* have reached high levels of genetic differentiation, high frequencies of M/S hybrids and very low levels of genetic differentiation between forms have been previously described in Guinea-Bissau (Oliveira et al., 2008). Additionally, the genes studied in this work are localized in the chromosome 3R (Rogers et al., 2009) explain why the differentiation between the two forms is not noticeable since M- and S- forms are significantly differentiated in three

regions of their genome - chromosomes X, 2L and 2R - called the genomic islands of speciation in *A. gambiae* (Turner et al., 2005) and more recently high diversity between M and S forms Mali was identified, adjacent to the centromeric region of chromosome 3L (White et al., 2010).

Both infected/non-infected mosquitoes and M- and S-form showed Ka/Ks ratio discordant for *AgTG1* and *AgTG2*, being higher than one for the *AgTG2* gene and lower than one for *AgTG1* gene. A ratio higher than one indicates positive selection (Ford, 2002) but the use of this ratio to detect positive selection is often not effective, because adaptive evolution most likely occurs at a few time points and at most times has an effect on only a few amino acids (Shen et al., 2012). In fact, when a codon-by-codon approach was used, some sites of *AgTG1* also presented Ka/Ks values higher than one, suggesting that some regions of this gene are under positive selection. Although not yet functionally characterized, Silveira et al. (2012) showed that *AgTG2* plays a role in containing *Plasmodium* infection. In this study, the authors show that there is an increase in both infection rate and intensity when mosquitoes are treated either with a TG inhibitor or with specific dsRNA and thus associating TG activity to protection against *Plasmodium*. Nsango et al. (2013) also found evidences of *AgTG2* involvement of *P. falciparum* killing in *A. gambiae*.

Positive selection was observed both associated to *Plasmodium* infection and molecular forms, suggesting that evolutionary pressure was not directly imposed by *Plasmodium*. Genes, such as *AgTG2* (Silveira et al., 2012), identified experimentally by gene-silencing to play a role in *Plasmodium* infection control might also be regulated by other microorganisms. The high levels of diversity found could be explained by the mosquito need to interact with a wide variety of pathogens, which corroborates by protein's 3D models, showing the majority of substitutions at periphery.

The comparison between several TG proteins sequences from different organisms can help to understand the evolution of TGs in different organisms and might help to infer the mosquito TG function. Lorand & Graham, (2003) draw a phylogenetic

analysis of papain-like TGs, and despite the high degree of sequence similarity between all of them, it was possible to distinguish at least to main branches: one that includes the genes for TG1, fXIIIA, invertebrates TGs and TG4; and other that includes genes for erythrocyte band 4.2, TG2, TG3, TG5, TG6 and TG7 (Lorand & Graham, 2003). In *Drosophila* sp., a single gene encodes for TG, and it is known that this enzyme is associated with the clot formation, which is an important effector of early innate immunity, preventing septic infections (Wang et al., 2010). The present study suggests AgTG1 and AgTG2 involvement in immune defence, as observed in *Drosophila*, *A. gambiae* and *A. stephensi* (Wang et al., 2010, Silveira et al., 2012).

In summary, data presented in this study did not indicate a clear differentiation for the two genes studied either between forms, or between infected and non-infected mosquitoes. Nevertheless, results strongly suggest that *AgTG1* and *AgTG2* have undergone adaptive evolution. The 3D model of the proteins showed that most sites under positive selection were found in the coding region of both genes; the majority of the substitutions are localized in the periphery where is more likely to interact with other molecules and therefore accumulate more variations. Finally, none of the mutations found, were located in critical sites for the protein function, as catalytic triads or in the amino acids residues potentially involved in calcium binding.

Although *AgTG1* and *AgTG2* exact function is not known, several studies in other organisms demonstrated that TGs are involved in a variety of immune responses: in *Drosophila* and in humans, TG accumulates on the surface of the microorganism, capturing it within the clot (Wang et al., 2010); the human coagulation factor XII mediates bacterial immobilization and killing inside the clot (Loof et al., 2011) and finally, the shrimp TG regulates immune-related genes, such as those that encode antimicrobial peptides (Fagutao et al., 2012). Also, Silveira et al. (2012) associated *AgTG2* to an increased resistance against *Plasmodium* infection. Present results point out to positive selection as the driven force of TGs evolution. Altogether, we hypothesize that these genes may play a role in pathogen recognition, being involved in the immune response triggered by the mosquito against the invading pathogens.

Authors' contributions

CM carried out the laboratory analysis and drafts the manuscript. APA, JP carried out the sampling and field data collection. AC and CM participated in the analysis and interpretation of data. CM, HS and APA drafted the paper. HS designed the study and participated in the analysis and interpretation of data. All authors read and approved the final manuscript.

Acknowledgements

We thank to the population of Antula, Bissau, who agreed to collaborate in this study. We thank the technicians Mário Gomes and João Dinis (Laboratório Nacional de Saúde Pública, Bissau), Marcelino Suna Nabion (Centro de Medicina Tropical, Bissau). This study was supported by PEst-OE/SAU/LA0018/2011 - Proj. Estratégico LA0018 2011/2012 (http://cmdt.ihmt.unl.pt/index.php/pt/); PTDC/SAU-MII/ 102596/2008 and PTDC/SAU-EPI/113326/2009, ‘‘Fundação para a Ciência e Tecnologia/Ministério da Educação e Ciência’’, FCT/MEC (http://alfa.fct.mctes.pt/index.phtml.pt), Portugal. C. Mendes and A. Custódio hold FCT grants (SRFH/BD/41473/2007 and BPD/IMUNOSTIM/MAL/2/2010, respectively).

References

Arez, A. P., Lopes, D., Pinto, J., Franco, A. S., Snounou, G. & do Rosário, V. E. 2000. *Plasmodium* sp.: Optimal protocols for PCR amplification from mosquito (*Anopheles* sp.) samples. *Exp Parasitol*, 94, 269-272.

Arez, A. P., Palsson, K, Pinto, K., Franco, A. S., Dinis, J., Jaenson, T. G. T., Snounou, G. & do Rosário, V. E. 1997. Transmission of mixed malaria species and strains by mosquitões, as detected by PCR, in a study area in Guinea-Bissau. *Parassitologia*, 39, 65-70.

Arez, A. P., Pinto, J., Pâlsson, K., Snounou, G., Jaenson, T. G. T. & do Rosário, V. 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum*. *Am J Trop Med Hyg*, 68,161-168.

Arnold, K., Bordoli, L., Kopp, J. & Schwede T. 2006. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling.

Barillas-Mury, C. 2007. CLIP proteases and *Plasmodium* melanization in Anopheles gambiae. *Trends in Parasitol*, 23, 297-299

Barreiro, L. B. & Quintana-Murci, L. 2010. From evolutionary genetics to human immunology: how selection shapes host defence genes. *Nature Rev Genet*, 11, 17-30.

Blandin, S. & Levashina, E. A. 2004. Mosquito immune responses against malaria parasites. *Curr Opin Immunol*, 16, 16-20.

Chirstophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N.T., Hoffmann, J.A., Kanzok, S.M., Letunic, I., Levashina, E.A., Loukeris, T.G., Lycett, G., Meister, S., Michel, K., Moita, L.F., Müller, H.M., Osta, M.A., Paskewitz, S.M., Reichhart, J.M., Rzhetsky, A., Troxler, L., Vernick, K.D., Vlachou, D., Volz, J., von Mering, C., Xu, J., Zheng, L., Bork, P. & Kafatos, F.C., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298, 159-165.

Clark, A. G., Glanowski, S., Nielsen, R., Thomas, P. D., Kejariwal, A., Todd, M. A., Tanenbaum, D. M., Civello, D., Lu, F., Murphy, B., Ferriera, S., Wang, G., Zheng, X., White, T. J., Sninsky, J. J., Adams, M. D. & Cargill, M. 2003. Inferring nonneutral evolution from human-chimp-mouse orthologous gene trios. *Science*, 302, 1960-1963.

Coetzee, M., Hunt, R. H., Wilkerson, R., Torre, A. D., Coulibaly, M. B. & Besansky, N. J. 2013. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the Anopheles gambiae complex. *Zootaxa*, 3619, 246-274.

Cohuet, A., Krishnakumar, S., Simard, F., Morlais, I., Koutsos, A., Fontenille, D., Mindrinos, M. & Kafatos, F. C. 2008. SNP discovery and molecular evolution in *Anopheles gambiae*, with special emphasis on innate immune system. *BMC Genomics*, 9, 227.

Crawford, J. E., Bischoff, E., Garnier, T., Gneme, A., Eiglmeier, K., Holm, I., Riehle, M. M., Guelbeogo, W. M., Sagnon, N., Lazzaro, B. P. & Vernick, K. D. 2012. Evidence for population-specific positive selection on immune genes of *Anopheles gambiae*. *3G*, 2, 1505-1519.

Excoffier, L., Smouse, P. E. & Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131, 479-491.

Excoffier, L., Laval, G. & Schneider, S. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol Bioinform Online*, 1, 47-50.

Fagutao, F. F., Maningas, M. B., Kondo, H., Aoki, T. & Hirono, I. 2012. Transglutaminase regulates immune-related genes in shrimp. Fish Shellfish *Immunol*, 32, 711-715.

Fanello, C., Santolamazza, F. & della Torre, A. 2002. Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Med Vet Entomol*, 16, 461-464.

Ford, M., 2002. Applications of selective neutrality tests to molecular ecology. *Mol Ecol*, 11, 1245-1262.

Fu, Y. & W. Li, 1993. Statistical tests of neutrality of mutations. *Genetics*, 133, 693-709.

Guex, N. & Peitsch, M. C. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modelling.

Haldane, J. B. S., 1949. Disease and evolution. *La Ricerca Scientifica* Supplemento A, 19, 68-76.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* (Oxf), 41, 95-98.

Hedrick, PW. 2012. Resistance to malaria in humans: the impact of strong, recent selection. *Malar J*, 11, 349.

Hudson, R. R., Slatkin, M. & Maddison, W. P. 1992. Estimation of Levels of Gene Flow from DNA Sequence Data. *Genetics*, 132, 583-589.

Jiggins, F. & Hurst, G. 2003. The evolution of parasite recognition genes in the innate immune system: purifying selection on *Drosophila melanogaster* peptidoglycan recognition proteins. *J Mol Evol*, 57, 598-605.

Jukes, T.H., & Cantor, C.R. 1969. Evolution of protein molecules. In: Mamalian protein metabolism. Munro, H.N. (Ed.). *Academic Press*, New York, pp.21-132.

Kumar, S., Nei, M., Dudley, J. & Tamura, K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform*, 9, 299-306.

Labate, J. A., Biermann, C. H. & Eanes, W. F. 1999. Nucleotide variation at the runt locus in *Drosophila melanogaster* and *Drosophila simulans*. *Mol Biol Evol*, 16, 724-731.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. & Higgins D. G. 2007. ClustalW and ClustalX version 2. *Bioinformatics*, 23, 2947-2948.

Le, B. V., Nguyen, J. B., Logarajah, S., Wang, B., Marcus, J., Williams, H. P., Catteruccia, F. & Baxter, R. H. G. 2013. Characterization of *Anopheles gambiae* transglutaminase 3 (AgTG3) and its native substrate Plugin. *J Biol Chem*, M112.435347

Lehmann, T., Hume, J. C., Licht, M., Burns, C. S., Wollenberg, K., Simard, F. & Ribeiro, J. M. 2009. Molecular evolution of immune genes in the malaria mosquito *Anopheles gambiae*. *PLoS One*, 4, e4549.

Librado, P. & Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451-1452.

Loof, T.G., Mörgelin, M., Johansson, L., Oehmcke, S., Olin, A., Dickneite, G., Norrby-Teglund, A., Theopold, U. & Herwald, H., 2011. Coagulation, an ancestral

serine protease cascade, exerts a novel function in early immune defence. *Blood*, 118, 2589-2598.

López, C., Saravia, C., Gomez, A., Hoebeke, J., Patarroyo, M. A. 2010. Mechanisms of genetically-based resistance to malaria. *Gene*. 467, 1-12.

Lorand, L. & Graham R. M., 2003. Transglutaminases: Crosslinking enzimes with pleiotropic functions. *Mol Cell Biol*, 4, 140-156.

Mendes, C., Felix, R., Sousa, A. M., Lamego, J., Charlwood D.,do Rosario, V. E., Pinto, J. & H. Silveira, 2010. Molecular evolution of the three short PGRPs of the malaria vectors *Anopheles gambiae* and *Anopheles arabiensis* in East Africa. *BMC Evol Biol*, 10, 9.

Mendes, C., Dias, F., Figueiredo, J., Mora, V. G., Cano, J., de Sousa, B., do Rosário, V. E., Benito, A., Berzosa, P. & Arez, A. P. 2011. Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis*, 5(6), e1192.

Mendes, C., Salgueiro, P., Gonzalez, V., Berzosa, P., Benito, A., do Rosário, V.E., de Sousa, B., Cano, J. & Arez A.P. 2013. Genetic diversity and signatures of selection of drug resistance in *Plasmodium* populations from both human and mosquito hosts in continental Equatorial Guinea. *Malar J*, 12, 114.

Morlais, I., Poncon, N., Simard, F., Cohuet, A. & Fontenille, D. 2004. Intraspecific nucleotide variation in *Anopheles gambiae*: New insights into the biology of malaria vectors. *Am J Trop Med Hyg*, 71, 795-802.

Nei, M., 1987. Molecular Evolutionary Genetics. *Columbia University Press*, New York, NY, USA.

Nielsen, R., Bustamante, C., Clark, A. G., Glanowski, S., Sackton T. B., Hubisz, M. J., Fledel-Alon, A., Tanenbaum, D. M., Civello, D., White, T. J., Sninsky, J. J., Adams, M. D. & Cargill, M. 2005. A scan for positively selected genes in the genomes of humans and chimpanzees. *PLoS Biol*, 3, e170

Nsango, S. E., Pompon, J., Xie, T., Rademacher, A., Fraiture, M., Thoma, M., Awono-Ambene, P. H., Moyou, R. S., Morlais, I. & Levashina, E. A. 2013. AP-1/Fos-TGase2 axis mediates wounding-induced *Plasmodium falciparum* killing in *Anopheles gambiae. J Biol Chem,* 288, 16145-16154.

Obbard, D. J., Callister, D. M., Jiggins, F. M., Soares, D. C., Yan, G. & Little, T. J. 2008. The evolution of TEP1, an exceptionally polymorphic immunity gene in *Anopheles gambiae*. *BMC Evol Biol*, 8, 274.

Obbard, D. J., Linton, Y. M., Jiggins, F. M., Yan, G. & Little, T. J. 2007. Population genetics of *Plasmodium* resistance genes in *Anopheles gambiae*: no evidence for strong selection. *Mol Ecol*, 16, 3497-3510.

Obbard, D. J., Welch, J. J. & Little, T. J. 2009. Inferring selection in the *Anopheles gambiae* species complex: an example from immune-related serine protease inhibitors. *Malar J*, 8, 117.

Oliveira, E., Salgueiro, P., Palsson, K., Vicente, J. L., Arez, A. P., Jaenson, T. G., Caccone, A. & Pinto J. 2008. High levels of hybridization between molecular

forms of *Anopheles gambiae* from Guinea Bissau. *J Med Entomol*, 45, 1057-1063.

Parmakelis, A., Slotman, M. A., Marshall, J. C., Awono-Ambene, P. H., Antonio-Nkondjio, C., Simard, F., Caccone, A. & Powell J. R. 2008. The molecular evolution of four anti-malarial immune genes in the *Anopheles gambiae* species complex. *BMC Evol Biol*, 8, 79.

Rogers, D. W., Baldini, F., Battaglia, F., Panico, M., Dell A, Morris, H. R. & Catteruccia, F. 2009. Transglutaminase-mediated semen coagulation controls sperm storage in the malaria mosquito. *PLoS Biol*, 7, e1000272.

Rottschaefer, S. M., Riehle, M. M., Coulibaly, B., Sacko, M., Niaré, O., Morlais, I., Traoré, S. F., Vernick, K. D. & Lazzaro, B. P. 2011. Exceptional diversity, maintenance of polymorphism, and recent directional selection on the APL1 malaria resistance genes of *Anopheles gambiae*. *PLoS Biol*, 9, e1000600.

Sackton, T. B., Lazzaro, B. P., Schlenke, T. A., Evans, J. D., Hultmark, D. & Clark, A. G. 2007 Dynamic evolution of the innate immune system in *Drosophila*. *Nat Genet,* 39, 1461-1468.

Schlenke, T. A. & Begun, D. J. 2003, Natural selection drives *Drosophila* immune system evolution. *Genetics*, 164, 1471-1480.

Schwede, T., Kopp, J., Guex, N. & Peitsch, M. C. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res*, 31, 3381-3385.

Shen, T., Xu, S., Wang, X., Yu, W., Zhou, K. & Yang, G.2012. Adaptive evolution and functional constraint at TLR4 during the secondary aquatic adaptation and diversification of cetaceans. *BMC Evol Biol*, 12, 39.

Shibata, T., Sekihara, S., Fujikawa, T., Miyaji, R., Maki, K., Ishihara, T., Koshiba, T. & Kawabata, S. 2013. Transglutaminase-catalyzed protein-protein cross-linking suppresses the activity of the NF-kB–Like transcription factor relish. *Sci Signal*, 6, ra61.

Silveira, S., Gabriel, A., Ramos, S., Palma, J., Félix, R., Custódio, A. & Collins, L. V. 2012. CpG-containing oligodeoxynucleotides increases resistance of *Anopheles* mosquitoes to *Plasmodium infection*. *Insect Bioch Mol Biol*, 42, 758-765.

Simard, F., Licht, M., Besansky, N. J. & Lehmann, T. 2007. Polymorphism at the defensin gene in the *Anopheles gambiae* complex: Testing different selection hypotheses. *Infect Genet Evol*, 7, 285-292.

Slotman, M. A., Parmakelis, A., Marshall, J. C., Awono-Ambene, P. H., Antonio-Nkondjo, C., Simard, F., Caccone, A. & Powell, J. R. 2007. Patterns of selection in anti-malarial immune genes in malaria vectors: evidence for adaptive evolution in LRIM1 in *Anopheles arabiensis*. *PLoS One*, 2, e793.

Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinheiro, L., do Rosário, V. E., Thaithong, S. & Brown, K. N. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol,* 1, 315-320.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics,* 123, 585-595.

Tiffin, P. & Moeller, D. A., 2006. Molecular evolution of plant immune system genes. *Trends Genet*, 22, 662-667.

Turner, T., Hahn, M. & Nuzhdin, S. 2005. Genomic islands of speciation in *Anopheles gambiae*. *PLoS Biol*, 3, e285.

Wang, Z., Wilhelmsson, C., Hyrsl, P., Loof, T. G., Dobes, P., Klupp, M., Loseva, O., Mörgelin, M., Iklé, J., Cripps, R. M., Herwald, H. & Theopold, U. 2010. Pathogen entrapment by transglutaminase – A conserved early innate immune mechanism. *Plos Pathog*, 6, e1000763.

White, B.J., Cheng, C., Simard, F., Costantini, C. & Besansky, N.J., 2010. Genetic association of physically unlinked islands of genomic divergence in incipient species of *Anopheles gambiae*. *Mol Ecol*, 19, 925-939.

Whitten, M. M. A., Shiao, S. W. & Levashina, E.A, 2006. Mosquito midguts and malaria: cell biology, compartimentalization and immunology. *Parasite Immunol*, 28, 121-130.

Wondji, C., Simard, F. & Fontenille, D. 2002. Evidence for genetic differentiation between the molecular forms M and S within the forest chromosomal form of *Anopheles gambiae* in an area of sympatry. *Insect Mol Biol*, 11, 11-19.

Additional file 1-table S1

Additional file 1 – Table S1 – Sequences and annealing temperatures of primers used to amplify the *AgTG1* and *AgTG2* genes of *A. gambiae*.

		Primer name	ID	Sequence	a.t.
AgTG1	**1st nest**	CMTGM100-1A-F	H	AATCACATGAATCCGCATGA	60ºC
		CMTGM100-1A-R		GGCGTTCATCTCCTCCATAA	
	2nd nest-5'	CMTGM100-1A-F	I	AATCACATGAATCCGCATGA	62ºC
		CMTGM100-2A-R		CAGCTTCGGTATCTTCCCACT	
	2nd nest-3'	TGM100-a-f	J	GCTGATGTGCTGACGGTAAA	62ºC
		TGM100-a-r		CCGAGAAGTCTCCACTCCAG	
	1stnest	TGM100-b-f	K	GTGTGTCGTGCCATCTCG	59ºC
		CMTGM100-1B-R		TGGCCCCTGTTTTTGTTTAG	
	2nd nest-5'	TGM100-b-f	L	GTGTGTCGTGCCATCTCG	58ºC
		TGM100-CM-R		CATTGTGGCCCGTTCTTC	
	2nd nest-middle	TGM100-c-F	M	AAGGATGTGCTTGGAATTGG	59ºC
		TGM100-c-R		CGGTGGTGTCATAGTGAACG	
	2nd nest-3'	CMTGM100-Y-F	N	GCAAACCGCTTCTTTTTAAGG	61ºC
		CMTGM100-Y-R		GGCCCCTGTTTTTGTTTAGC	
AgTG2	**1st nest**	CMTGM98-1A-F	A	ATAGGCCACACCTCACCTTG	62 ºC
		CMTGM98-1A-R		TTCCCTTCCAGATCATCGTC	
	2nd nest-5'	CMTGM98-1A-F	B	ATAGGCCACACCTCACCTTG	63ºC
		CMTGM98-2-A-R		CGCCGAAATGTCTTACCATT	
	2nd nest-3'	CMTGM98-2-B-F	C	TGCTGGCGCTGTATAAAATG	59ºC
		CMTGM98-1A-R		TTCCCTTCCAGATCATCGTC	
	1st nest	CMTGM98-1B-F	D	GGAGTGGAGGAGAGCAACAG	60ºC
		CMTGM98-1B-R		TCTCTGAGGATCGAGGAAGC	
	2nd nest-5'	TGM98-CM-F	E	TCAATGCCTGGTGTGAAGAG	62ºC
		TGM98-CM-R		CCGCAAAGACAAAGTCACAA	
	2nd nest-middle	CMTGM98-2D-F	F	AACTACCACGTGTGGAACGA	65ºC
		CMTGM98-2D-R		GCGAAGCTGGTCACCTTAAA	
	2nd nest-3'	CMTGM98-Z- F	G	ACGGGCAAGAACATCAAAAC	59ºC
		CMTGM98-Z- R		AATGGCCGATAAAACAGGTG	

a.t. – annealing temperature

Additional file 2-table S2

Additional file 2 – Table S2 – Matrix of pairwise comparisons of Fst for the two populations and for the two groups of infection studied.

		Non-infected	**Infected**	***A. gambiae*_M**	***A. gambiae*_S**
AgTG1	**Non-infected**	-			
	Infected	0.007 ^{NS}	-		
	***A. gambiae*_M**	-0.049 ^{NS}	-0.026 ^{NS}	-	
	***A. gambiae*_S**	-0.031 ^{NS}	-0.048 ^{NS}	0.005 ^{NS}	-
AgTG2	**Non-infected**	-			
	Infected	0.003 ^{NS}	-		
	***A. gambiae*_M**	-0.045 ^{NS}	-0.037 ^{NS}	-	
	***A. gambiae*_S**	-0.032 ^{NS}	-0.044 ^{NS}	0.004 ^{NS}	-

^{NS} – not significant

Additional file 3-table S3

Additional file3 – Table S3– Hierarchical analysis of molecular variance (AMOVA) among the infected and non-infected groups.

Source of variation	Hierarchical AMOVA for infected and non-infected groups	
	AgTG1	***AgTG2***
Among groups	-0.35	-4.37
Among populations within groups	5.77	0.40
Within populations	94.59	103.98
Fcs (population/group)	0.057^{NS}	0.004^{NS}
Fst (population/total)	0.054^{NS}	-0.040^{NS}
Fct (group/total)	-0.004^{NS}	-0.043^{NS}

^{NS} – not significant

Additional file 4-table S4

Additional file4 – Table S4– Hierarchical analysis of molecular variance (AMOVA) among the *A. gambiae* S-form and *A. gambiae* M-form groups.

Source of variation	**Hierarchical AMOVA for *A. gambiae* S-form and *A. gambiae* M-form**	
	AgTG1	***AgTG2***
Among groups	-2.00	0.15
Among populations within groups	6.89	0.99
Within populations	95.12	98.85
Fcs (population/group)	0.100^{NS}	0.379
Fst (population/total)	0.066^{NS}	0.303
Fct (group/total)	1.000^{NS}	0.670

^{NS} – not significant

Chapter 5 – General Discussion and Conclusions

5. General discussion and conclusions

Despite of decades of research and efforts in its combat, malaria continues to be one of the major public health problems worldwide, affecting all tropical and subtropical areas of the planet, as some African countries like Equatorial Guinea, Angola and Guinea Bissau, where the disease is one of the main causes of child mortality and morbidity.

The main objectives of this thesis were to analyse the complex malaria system under a comprehensive approach. We studied the diversity of *Plasmodium* populations circulating in both mosquito vector and human host in the same area at the household level. Further, we investigated the selective signatures, origin and spread of antimalarial resistance associated mutations in the *Plasmodium* genome as well as genetic diversity patterns of selection and evolution of mosquito immune related genes and its possible relationship with infection.

Ultimately, present study also contributed to update the knowledge about the malaria epidemiology in mainland Equatorial Guinea. This area characterization may lead to the improvement of malaria control programs. This study provided crucial data for the correct management of malaria cases in the area, and in particular, provided precise information on the population structure of *Plasmodium* parasites, with special relevance on the selection and dispersal of drug resistance genes, which is of utmost importance in designing and implementing therapeutic policies at a national basis.

The first aim of this study was the characterization of the circulating populations of *Plasmodium* spp., in Equatorial Guinea, through a combined set of human peripheral blood and mosquito samples collected in both coastal and inland villages. The collected data was of major importance, because there was no prior information about malaria transmission and prevalence of *Plasmodium* species in the mainland, although much of this information was already available to the islands (Pardo et al., 2006; Kleinschmidt et al., 2009).

In the mainland, the detection and identification of *Plasmodium* was made both in humans and mosquito vectors and results showed a higher prevalence of *Plasmodium* infection than was expected when comparing with the results obtained for the island (87% *versus* 32%) (Kleinschmidt et al., 2007). However, those differences are justified considering that most of malaria control activities have been carried out in the island of Bioko where the capital Malabo is located. These differences are a great example of how control efforts have a tangible impact on malaria transmission. In the insular region of the Equatorial Guinea after the implementation of the BIMCP, the prevalence of infection was reduced from 42% pre-intervention to 18% in 2008 (Pardo et al., 2006; Kleinschmidt et al., 2009); in Angola, and according to the last report of the President's Malaria Initiative (2013), the prevalence of malaria has dropped 50% over the last five years as a result of control efforts.

The data obtained in this study highlight the necessity of the implementation of control measures such as, vector control (*e.g*. chemical spraying) and personal protection/prevention strategies (*e.g.* ITNs, the use of effective anti-malarial drugs – presently ACT is recommend, in this case oral AS with AQ), in the mainland.

The characterization of the circulating populations of *Plasmodium* spp., in the set of human peripheral blood and mosquito samples showed the presence of four species, being *P. falciparum* the most prevalent, occurring in 90% of the isolates (both humans and mosquitoes). Knowing that the study of genetic diversity in natural populations of *Plasmodium* is essential from an epidemiological point of view, since ecological interactions between parasite populations in the same host may be an important source of selection on drug resistance genes for example, the *P. falciparum* diversity was analysed using an antigen coding gene - *Pfmsp2* - as well as a set of neutral STR *loci.*

High levels of genetic diversity were found, when *P. falciparum* diversity were analysed in both hosts, which is indicative of high levels of malaria endemicity in mainland Equatorial Guinea; and similar population genetic structure of parasites both in humans and mosquitoes, which is concordant with other studies in African malaria highly endemic countries (Babiker et al., 1995; Anderson et al., 2000).

When comparison of parasite genetic diversity was made between the two hosts, between the two locations and between the two seasons, no significant genetic differentiation was found, which may indicate consistency in the parasite populations that are being transmitted, and may indicate that despite the ~60Km that separate Ngonamanga from Miyobo, there are no evidences for the existence of barriers to gene flow, parasite migration, and other possible influences on population structure.

These results are of major importance, since levels of diversity are an indication of the fitness of the parasite population and thus how difficult it could be to target it with drugs or vaccines.

Another important result was the finding of both humans and mosquitoes infected with *P. vivax*. This was an unexpected result, mainly because this parasite is virtually absent in West and Central Africa, due to the high prevalence of Duffy negative individuals [Fy(a^-b^-)], described as being resistant to the infection by this parasite (Miller et al., 1975; Langhi & Bordin 2006). To increase the strength of the study, samples from Angola (another country where the presence of *P. vivax* is extremely low and the prevalence of Duffy negative individuals is very high) were included in the present investigation.

In both countries, Duffy negative people were found to be infected with *P. vivax*. Similar results have been previously found in other countries, like Kenya - East Africa (Ryan et al., 2006), Amazon region in Brazil (Cavasini et al., 2007a; Cavasini et al., 2007b) and, more recently, in Madagascar (Ménard et al., 2010), but those are areas where the prevalences of Duffy positive are significantly high.

The presence and/or absence of *P. vivax* in Africa is not a consensus theme. In fact several authors raise questions about the emergence of the Duffy negative allele in Africa, when is believed that *P. vivax* has had his origin in Asia (Liu et al., 2014) or why several reports of persons infected with *P. vivax* returning from areas with high prevalences of Duffy negative are frequently arising (Poirriez et al., 1991; Gautret et al., 2001; Muhlberger et al., 2004) suggesting the existence of active transmission of this species.

This study demonstrated the existence of active transmission of *P. vivax* in Equatorial Guinea, being this parasite present both in mosquito and human populations.

Seems to be unquestionable that this specie is evolving and is able to use other receptors than Duffy to invade the erythrocytes and this capacity is not exclusive for one strain of *P. vivax*, since in this study we were able to found samples infected with both *P. vivax* VK247 and *P. vivax* VK210 (also called *P. vivax* classic) (Mendes et al., 2011), but is this the only explanation for existence of this *Plasmodium* specie in areas where the prevalence of Duffy negatives is near 100%?

Some authors suggest that the small prevalence of Duffy positive individuals (~1%-5%) might be enough to maintain the transmission of *P. vivax* (Culleton et al., 2008); others point to a possible existence of a zoonotic reservoir of this parasite (Culleton & Carter, 2012; Prugnolle et al., 2013). Prugnolle and collaborators (2013), showed, through the analysis of complete mitochondrial genome, that despite *P. vivax* sequences from parasites of great apes forms a genetically distinct clade from parasites that circulates in humans, the parasites from the great apes clade can be infectious to humans. Other studies (Kaiser et al., 2010; Kreif et al., 2010; Liu et al., 2010) also proved that parasites very closed related to *P. vivax* of humans are very prevalent in non-human hosts in central West Africa.

These findings may help to explain the presence of this parasite in areas with high prevalences of Duffy negative individuals, but do not diminish the results found in this study where it is shown that this parasite is evolving, being able to infect Duffy negative people and it seems perfectly adapted to new environments being found in areas where it was not previously described, like Congo (Gautret et al., 2001), São Tomé and Principe (Snounou et al., 1998; Gautret et al., 2001), Gabon (Poirriez et al., 1991; Gautret et al., 2001) and Cameroon (Gautret et al., 2001).

In conclusion, this work helped to reinforce the idea that *P. vivax* might become a very serious public health problem in West and Central Africa, once it is capable of causing more severe forms of the disease including death (Genton et al., 2008; Rogerson & Carter, 2008; Tjitra et al., 2008; Kochar et al., 2009; Alexandre et al., 2010). It is important that these new findings are taken into account when setting new control measures and implementation of prophylactic or therapeutic regimens.

The parasite resistance *status* to antimalarial drugs is always a crucial point because it still is the main caveat to malaria control, mostly in countries like Equatorial

Guinea, where available data is scarce. To try to compensate this poor knowledge about parasite resistance to drugs, four antimalarial resistance associated genes - *Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps* - were also studied. Additionally, to trace the origin and progression of antimalarial resistance in this country, six STRs flanking *Pfdhfr* and *Pfdhps* genes were also analysed.

Despite the CQ no longer be administered within the national therapeutic guidelines, the prevalence of the main point mutations associated with resistance to this drug (75E and 76T of *Pfcrt* gene and 86Y of *Pfmdr1* gene) remains very high, reaching 63%. With the absence of drug pressure would be expected that the prevalence of mutations associated with resistance to this drug would decrease, as reported in other countries (Kublin et al., 2003; Wang et al., 2005; Mwai et al., 2009; Fançony et al., 2012), however, a recent study conducted in Equatorial Guinea (Amor et al., 2012) found higher prevalence of mutation 76T of *Pfcrt* gene and 1246Y of *Pfmdr1*gene, than those found in this study. This increment might be a result of selective pressure by AS/AQ combination, since AQ is a close Mannich base analogue of CQ, promoting the maintenance of CQ-resistant isolates with the mutant *Pfcrt* and *Pfmdr1* genotypes. On the other hand, other possibility is the continuous use of CQ despite the national therapeutic guidelines (Plowe et al., 1995).

Regarding SP resistance, it is known that the continuous use of this drug leads to a rapidly increase of the resistance levels. In Equatorial Guinea this drug has been used for many years as a second therapeutic line and even today it is used in preventive chemotherapy in pregnancy. Therefore, it is not surprising the high frequencies found: approximately 70%, for the SP combination.

In fact, the PYR resistance seems to be well established in mainland Equatorial Guinea with nearly 80% of the parasite populations presenting the triple mutant N51I/C59R/S108N in the *Pfdhfr* gene. On the other hand, for SFX resistance, a high prevalence of the mutation A437G in *Pfdhps* was detected; however the mutation K540E was practically non-existent, as expected for countries from West Africa (Pearce et al., 2009). Despite the results described above, no samples were found containing the quintuple mutant, associated with SP clinical failure (Kublin et al., 2002; Talisuna et al., 2004). These results are highly alarming since this drug is the only antimalarial

medicine for which data on efficacy and safety for IPT is available from controlled clinical trials (WHO, 2013).

Data on evolutionary origin and rate of spread of drug resistance-conferring mutations have important implications for drug policies implementation, and the analysis of flanking genetic markers can importantly contribute to this knowledge.

Flanking STRs of the genes *Pfdhfr* and *Pfdhps* were analysed. It was expected that the extensive use of SP would lead to a rapid increase of the resistance levels, as seen in other countries like Cameroon (Tahar & Basco, 2006), Gabon (Aubouy et al., 2003) and São Tomé and Principe (Salgueiro et al., 2010); leaving signatures of drug selective pressure. In fact, in the *loci* flanking *Pfdhfr* gene, a reduction in heterozigosity (*He)* was observed suggesting that this gene has undergone strong selection in Equatorial Guinea. This result together with a higher mean of *He* around double mutant than around the triple mutant are in accordance with a model of positive directional selection. For the *loci* flanking *Pfdhps* gene, the remarkably high values of *He* suggests the presence of multiple lineages occurring within individual populations, and significant LD values were found. These results might be suggestive of soft selective sweep, where multiple lineages are superimposed within a single population causing higher *He* values than in populations where a single lineage is present (Nair et al., 2007).

The results obtained for the *loci* flanking *Pfdhfr* gene showed that in Ngonamanga the majority of the haplotypes found were associated with the triple mutants, while in Miyobo the majority was associated with double mutants. This suggests that PYR resistance was firstly established in Ngonamanga, probably due to their "proximity" to the Malabo (the capital of the country), where the introduction of the drug may have started, and later in Miyobo, a more isolated area. Similar results were obtained for the *loci* flanking *Pfdhps* gene, which indicate that the resistance to SFX have also been established earlier in Ngonamanga, where the prevalence of triple mutants is higher than in Miyobo.

In conclusion, it was possible to confirm that CQ should no longer be recommended for malaria treatment in Equatorial Guinea, since high frequency of mutations associated with the resistance to this drug were observed. On the other hand,

and despite the high frequency found for some mutations associated with the decrease in the efficacy of SP, namely the triple mutant A436/G437/G581, this drug remains in use in combination with AS and it is the only drug recommended for intermittent preventive therapy in pregnancy (Kleinschmidt et al., 2006). Considering these results, it is recommended a close and continuous monitoring of point mutations frequency in the two genes associated with SP resistance, *Pfdhfr* and *Pfdhps*, since there is the danger of an eventual reduction in the efficacy of SP combined therapy.

Finally, this study also addressed the study of mosquito immune response to *Plasmodium* infection. In fact this issue has become relevant in the last years since its manipulation could be used to break transmission. Currently it is known that mosquitoes respond differently according to their genetic background and infectious *Plasmodium* species, but the major information was gathered through malaria experimental models and data on naturally infected mosquitoes is still scarce. On an attempt to overcome this, this work relied on well characterized subpopulations of infected/non infected mosquitoes from Guinea-Bissau (already available in our laboratory from previous studies). It is important to explain that mosquitoes from Equatorial Guinea were not used in this part of the work due to limitations in the number of successfully amplified sequences.

In this work two immune related genes – *AgTG1* and *AgTG2* – were analysed to try to established their patterns of evolution and determine if the *Plasmodium* exerted some kind of selection pressure on them.

Results showed that these genes presented high levels of nucleotide diversity which is in agreement with other studies that analysed other immune related genes in *A. gambiae* (Morlais et al., 2004; Simard et al., 2007; Cohuet et al., 2008; Mendes et al., 2010). When the comparison between the M- and S- forms of *A. gambiae* mosquitoes was made, no significant differences were found, suggesting that these two forms do not present significant genetic differentiation in these genes, in this sampling location. Despite other studies, like the ones developed by Crawford and collaborators (2012) and by Wondji and collaborators (2002), who found that molecular forms of *A. gambiae* have reached high levels of genetic differentiation, a study conducted also in the Guinea

Bissau, reported high frequencies of M/S hybrids and very low levels of genetic differentiation between forms suggesting that the degree of isolation between M- and S-forms, is not homogenous throughout the species distribution range (Oliveira et al., 2008).

The *AgTG2* gene presented remarkable high levels of nucleotide diversity in the nonsynonymous sites. These values despite of being 10x higher than the *A. gambiae* genome average diversity, are in concordance with other studies that show genes evolving under positive selection, revealing evidence of host-pathogen co-evolutionary dynamics (Obbard et al., 2008; Rottschaefer et al., 2011). These results were corroborated with Ka/Ks ratio that presented values higher than one, indicating that this gene is under positive selection (Ford, 2002). For the *AgTG1*, a codon by codon approach was used, and some regions of this gene presented Ka/Ks values higher than one in some points of the gene, which suggests that some regions of this gene are also under positive selection.

When proteins encoded by these genes were analysed, high levels of diversity were found. This might be due to the need of the mosquito vector to recognize a wide variety of pathogens, and in fact, when 3D models were developed, it was clear that the substitutions were concentrated at the periphery which agrees with this hypothesis.

In conclusion, the data presented in this study strongly suggest that *AgTG1* and *AgTG2* have undergone adaptive evolution, with some regions of the genes under positive selection. Understanding the biological mechanisms underlying this positive selection is beyond of the objectives of work, and although the exact function of the two TGs in regarding infection is not clear, it is possible that *AgTG1* and *AgTG2* have slightly differences in their functions. Although *AgTG2* shows stronger signs of positive selection when compared with *AgTG1,* both genes seems to play a role in the recognition of pathogens, being also involved in the immune response triggered by the mosquito. More studies about these proteins are needed, to know their exact involvement in the defence against *Plasmodium* and/or other microorganisms.

This study, through the analysis of all entities involved in the malaria transmission, is a good way to quickly find new strategies to control it. In fact, one of

the main caveats to the malaria control is the resistance developed by both parasites and mosquito vectors to antimalarial drugs and insecticides, respectively.

The present work contributed to a better description about: malaria transmission, parasite resistance to anti-malarial drugs, evolutionary origin and rate of spread of drug resistance of SP in the mainland of Equatorial Guinea and mosquito immune response to *Plasmodium* infection. Thus, the gained knowledge with this work will certainly be important on the long run sustainability of malaria control.

References

Alexandre, M. A., Ferreira, C. O., Siqueira, A. M., Magalhães, B. L., Mourão, M. P., Lacerda, M. V. & Alecrim, Md. 2010. Severe *Plasmodium vivax* Malaria, Brazilian Amazon. *Emerg Infect Dis*, 16, 1611-1614.

Amor, A., Toro, C., Fernández-Martínez, A., Baquero, M., Benito, A. & Berzosa, P. 2012. Molecular markers in *Plasmodium falciparum* linked to resistance to anti-malarial drugs in samples imported from Africa over an eight-year period (2002-2010): impact of the introduction of artemisinin combination therapy. *Malar J*, 11, 100.

Anderson, T. J., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L., Mollinedo, R., Bockarie, M., Mokili, J., Mharakurwa, S., French, N., Whitworth, J., Velez, I. D., Brockman, A. H., Nosten, F., Ferreira, M. U. & Day, K. P. 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *P. falciparum*. *Mol Biol Evol*, 17, 1467-1482.

Aubouy, A., Jafari, S., Huart, V., Migot-Nabias, F., Mayombo, J., Durand, R., Bakary, M., Le Bras, J. & Deloron, P. 2003. DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with in vitro activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. *J Antimicrob Chemother*, 52, 43-49.

Babiker, H. A., Satti, G. & Walliker, D. 1995. Genetic changes in the population of *Plasmodium falciparum* in a Sudanese village over a three-year period. *Am J Trop Med Hyg*, 53, 7-15.

Cavasini, C. E., de Mattos, L. C., Couto, A. A., Couto, V. S., Gollino, Y., Moretti, L. J., Bonini-Domingos, C. R., Rossit, A. R., Castilho, L. & Machado, R. L. 2007b. Duffy blood group gene polymorphisms among malaria *vivax* patients in four areas of the Brazilian Amazon region. *Malar J,* 6, 167-175.

Cavasini, C. E., Mattos, L. C., Couto, A. A., Bonini-Domingos, C. R., Valencia, S. H., Neiras, W. C., Alves, R. T., Rossit, A. R., Castilho, L. & Machado, R. L. 2007a. *Plasmodium vivax* infection among Duffy antigen-negative individuals from the Brazilian Amazon region: an exception? *Trans R Soc Trop Med Hyg,* 101, 1042-1044.

Cohuet, A., Krishnakumar, S., Simard, F., Morlais, I., Koutsos, A., Fontenille, D., Mindrinos, M. & Kafatos, F. C. 2008. SNP discovery and molecular evolution in *Anopheles gambiae*, with special emphasis on innate immune system. *BMC Genomics*, 9, 227.

Crawford, J. E., Bischoff, E., Garnier, T., Gneme, A., Eiglmeier, K., Holm, I., Riehle, M. M., Guelbeogo, W. M., Sagnon, N., Lazzaro, B. P. & Vernick, K. D. 2012. Evidence for population-specific positive selection on immune genes of *Anopheles gambiae. G3*, 2, 1505-1519.

Culleton, R. & Carter, R. 2012. African *Plasmodium vivax*: distribution and origins. *Int J Parasitol*, 42:1091-1097.

Culleton, R. L., Mita, T., Ndounga, M., Unger, H., Cravo, P. V., Paganotti, G. M., Takahashi, N., Kaneko, A., Eto, H., Tinto, H., Karema, C., D'Alessandro, U., do Rosário, V., Kobayakawa, T., Ntoumi, F., Carter, R. & Tanabe, K. 2008. Failure to detect *Plasmodium vivax* in West and Central Africa by PCR species typing. *Malar J*, 7, 174.

Fançony, C., Gamboa, D., Sebastião, Y., Hallett, R., Sutherland, C., Sousa-Figueiredo, J. C. & Nery, S. V. 2012. Various *pfcrt* and *pfmdr1* genotypes of *Plasmodium falciparum* cocirculate with *P. malariae*, *P. ovale* spp., and *P. vivax* in Northern Angola. *Antimicrob Agents Chemother*, 56, 5271-5277.

Ford, M., 2002. Applications of selective neutrality tests to molecular ecology. *Mol Ecol,* 11, 1245-1262.

Gautret, P., Legros, F., Koulmann, P., Rodier, M. H. & Jacquemin, J. L. 2001. Imported *Plasmodium vivax* malaria in France: geographical origin and report of an atypical case acquired in Central or Western Africa. *Acta Trop,* 78, 177-181.

Genton, B., D'Acremont, V., Rare, L, Baea, K., Reeder, J. C., Alpers, M. P. & Müller, I. 2008. *Plasmodium vivax* and mixed infections are associated with severe malaria in children: A prospective cohort study from Papua New Guinea. *PLoS Med,* 5, 881-889.

Kaiser, M., Löwa, A., Ulrich, M., Ellerbrok, H., Goffe, A.S., Blasse, A., Zommers, Z., Couacy-Hymann, E., Babweteera, F., Zuberbühler, K., Metzger, C., Geidel, S., Boesch, C., Gillespie, T.R. & Leendertz, F.H., 2010. Wild chimpanzees infected with five *Plasmodium* species. *Emerg Infect Dis,* 16, 1956-1959.

Kleinschmidt, I., Schwabe, C., Benavente, L., Torrez, M., Ridl, F. C., Segura, J. L., Ehmer, P. & Nchama, G. N. 2009. Marked increase in child survival after four years of intensive malaria control. *Am J Trop Med Hyg*, 80:882-888.

Kleinschmidt, I., Sharp, B., Benavente, L. E., Schwabe, C., Torrez, M., Kuklinski, J., Morris, N., Raman, J. & Carter, J. 2006. Reduction in infection with *Plasmodium falciparum* one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea. *Am J Trop Med Hyg*, 12, 972-978.

Kleinschmidt, I., Torrez, M., Schwabe, C., Benavente, L., Seocharan, I., Jituboh, D., Nseng, G. & Sharp, B. 2007. Factors influencing the effectiveness of malaria control in Bioko Island, Equatorial Guinea. *Am J Trop Med Hyg*, 76, 1027-1032.

Kochar, D. K., Das, A., Kochar, S. K., Saxena, V., Sirohi, P., Garg, S., Kochar, A., Khatri, M. P. & Gupta, V. 2009. Severe *Plasmodium vivax* malaria: a report on serial cases from Bikaner in northwestern India. *Am J Trop Med Hyg*, 80, 194-198.

Krief, S., Escalante, A.A., Pacheco, M.A., Mugisha, L., André, C., Halbwax, M., Fischer, A., Krief, J.M., Kasenene, J.M., Crandfield, M., Cornejo, O.E., Chavatte, J.M., Lin, C., Letourneur, F., Grüner, A.C., McCutchan, T.F., Rénia, L., Snounou, G., 2010. On the diversity of malaria parasites in African apes and the origin of *Plasmodium falciparum* from Bonobos. *PLoS Pathog*, 6, e1000765.

Kublin, J. G., Cortese, J. F., Njunju, E. M., Mukadam, R. A., Wirima, J. J., Kazembe, P. N., Djimdé, A. A., Kouriba, B., Taylor, T. E & Plowe, C. V. 2003. Reemergence

of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infect Dis*, 187, 1870-1875.

Kublin, J. G., Dzinjalamala, F. K., Kamwendo, D. D., Malkin, E. M., Cortese, J. F., Martino, L. M., Mukadam, R. A., Rogerson, S. J., Lescano, A. G., Molyneux, M. E., Winstanley, P. A., Chimpeni, P., Taylor, T. E. & Plowe, C. V. 2002. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis*, 185, 380-388.

Langhi, D. M. & Jr., Bordin, J. O. 2006. Duffy blood group and malaria. *Hematology*, 11, 389-398.

Liu, W., Li, Y., Learn, G.H., Rudicell, R.S., Robertson, J.D., Keele, B.F., Ndjango, J.B., Sanz, C.M., Morgan, D.B., Locatelli, S., Gonder, M.K., Kranzusch, P.J., Walsh, P.D., Delaporte, E., Mpoudi-Ngole, E., Georgiev, A.V., Muller, M.N., Shaw, G.M., Peeters, M., Sharp, P.M., Rayner, J.C. & Hahn, B.H., 2010. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature 467, 420-425.

Liu, W., Li, Y., Shaw, K. S., Learn, G. H., Plenderleith, L. J., Malenke, J. A., Sundararaman, S. A., Ramirez, M. A., Crystal, P. A., Smith, A. G., Bibollet-Ruche, F., Ayouba, A.,Locatelli, S., Esteban, A., Mouacha, F., Guichet, E., Butel, C., Ahuka-Mundeke, S., Inogwabini, B. I., Ndjango, J. B., Speede, S., Sanz, C. M., Morgan, D. B., Gonder, M. K., Kranzusch, P. J., Walsh, P. D., Georgiev, A. V., Muller, M. N., Piel, A. K., Stewart, F. A., Wilson, M. L., Pusey, A. E., Cui, L., Wang, Z., Färnert, A., Sutherland, C. J., Nolder, D., Hart, J. A., Hart, T. B., Bertolani, P., Gillis, A., LeBreton, M., Tafon, B., Kiyang, J., Djoko, C. F., Schneider, B. S., Wolfe, N. D.,Mpoudi-Ngole, E., Delaporte, E., Carter, R., Culleton, R. L., Shaw, G. M., Rayner, J. C., Peeters, M., Hahn, B. H. & Sharp, P. M. 2014. African origin of the malaria parasite *Plasmodium vivax*. *Nat Commun*, 5, 3346.

Ménard, D., Barnadas, C., Bouchier, C., Henry-Halldin, C., Gray, L. R., Ratsimbasoa, A., Thonier, V., Carod, J. F., Domarle, O., Colin, Y., Bertrand, O., Picot, J., King, C. L., Grimberg, B. T., Mercereau-Puijalon, O. & Zimmerman, P. A. 2010. *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proc Natl Acad Sci USA*, 107, 5967-5971.

Mendes, C., Dias, F., Figueiredo, J., Mora, V. G., Cano, J., de Sousa, B., do Rosário, V. E., Benito, A., Berzosa, P. & Arez, A. P. 2011. Duffy negative antigen is no longer a barrier to *Plasmodium vivax* – molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis*, 5: e1192.

Mendes, C., Felix, R., Sousa, A.M., Lamego, J., Charlwood, D., do Rosario, V., Pinto, J. & Silveira, H. 2010. Molecular evolution of the three short PGRPs of the malaria vectors *Anopheles gambiae* and *Anopheles arabiensis* in East Africa. *BMC Evol Biol*, 10, 9.

Miller, L. H., Mason, S. J, Dvorak, J. A., McGinniss, M. H. & Rothman, I. K. 1975. Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science*, 189, 561-563.

Mobegi, V. A., Loua, K. M., Ahouidi, A. D., Satoguina, J., Nwakanma, D. C., Amambua-Ngwa, A. & Conway, D. J. 2012. Population genetic structure of

Plasmodium falciparum across a region of diverse endemicity in West Africa. *Malar J*, 11, 223.

Morlais, I., Poncon, N., Simard, F., Cohuet, A. & Fontenille, D. 2004. Intraspecific nucleotide variation in *Anopheles gambiae*: New insights into the biology of malaria vectors. *Am J Trop Med Hyg*, 71, 795-802

Muhlberger, N., Jelinek, T., Gascon, J., Probst, M., Zoller, T., Schunk, M., Beran, J., Gjørup, I., Behrens, R. H., Clerinx, J., Björkman, A., McWhinney, P., Matteelli, A., Lopez-Velez, R., Bisoffi, Z., Hellgren, U., Puente, S., Schmid, M. L., Myrvang, B., Holthoff-Stich, M. L., Laferl, H., Hatz, C., Kollaritsch, H., Kapaun, A., Knobloch, J., Iversen, J., Kotlowski, A., Malvy, D. J., Kern, P., Fry, G., Siikamaki, H., Schulze, M. H., Soula, G., Paul, M., Gómez i Prat, J., Lehmann, V., Bouchaud, O., da Cunha, S., Atouguia, J. & Boecken, G. 2004. Epidemiology and clinical features of vivax malaria imported to Europe: sentinel surveillance data from TropNetEurop. *Malar J*, 3:5.

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S. M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann, S., Mackinnon, M. & Nzila, A. 2009. Chloroquine resistance before and after its withdrawal in Kenya. *Malar J*, 8, 106.

Nair, S., Nash, D., Sudimack, D., Jaidee, A., Barends, M., Uhlemann, A. C., Krishna, S., Nosten, F. & Anderson, T. J. 2007. Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites. *Mol Biol Evol,* 24, 562-573.

Obbard, D. J., Callister, D. M., Jiggins, F. M., Soares, D. C., Yan, G. & Little, T. J. 2008. The evolution of TEP1, an exceptionally polymorphic immunity gene in *Anopheles gambiae*. *BMC Evol Biol,* 8, 274.

Oliveira, E., Salgueiro, P., Palsson, K., Vicente, J.L., Arez, A.P., Jaenson, T.G., Caccone, A. & Pinto J., 2008. High Levels of hybridization between molecular forms of *Anopheles gambiae* from Guinea Bissau. *J Med Entomol*, 45, 1057-1063.

Pardo, G., Descalzo, M. A., Molina, L., Custodio, E., Lwanga, M., Mangue, C., Obono, J., Nchama, A., Roche, J., Benito, A., Cano, J. 2006. Impact of different strategies to control Plasmodium infection and anaemia on the island of Bioko (Equatorial Guinea). *Malar J*, 5:10.

Pearce, R. J., Pota, H., Evehe, M. S., Bâ, e-H., Mombo-Ngoma, G., Malisa, A. L., Ord, R., Inojosa, W., Matondo, A., Diallo, D. A., Mbacham, W., van den Broek, I. V., Swarthout, T. D., Getachew, A., Dejene, S., Grobusch, M. P., Njie, F., Dunyo, S., Kweku, M., Owusu-Agyei, S., Chandramohan, D., Bonnet, M., Guthmann, J. P., Clarke, S., Barnes, K. I., Streat, E., Katokele, S. T., Uusiku, P., Agboghoroma, C. O., Elegba, O. Y., Cissé, B., A-Elbasit, I. E., Giha, H. A., Kachur, S. P., Lynch, C., Rwakimari, J. B., Chanda, P., Hawela, M., Sharp, B., Naidoo, I. & Roper, C. 2009. Multiple origins and regional dispersal of resistant dhps in African *Plasmodium falciparum* malaria. *PLoS Med*, 6, e1000055.

Plowe, C. V. & Wellems, T. E. 1995. Molecular approaches to the spreading problem of drug resistant malaria. *Adv Exp Med Biol*, 390, 197-209.

Poirriez, J., Landau, I., Verhaeghe, A., Savage, A. & Dei-Cas, E. 1991. Atypical forms of *Plasmodium vivax*. Apropos of a case. *Ann Parasitol Hum Comp*, 66, 149-154.

President's Malaria Initiative, Angola, Malaria Operational Plan FY 2013. USAID (http://www.pmi.gov/docs/default-source/default-document-library/country-profiles/angola_profile.pdf?sfvrsn=22, accessed in October 20th, 2013).

Price, R. N., Tjitra, E., Guerra, C. A., Yeung, S., White, N. J. & Anstey, N. M. 2007. *Vivax* malaria: neglected and not benign. *Am J Trop Med Hyg,* 77, 79-87.

Prugnolle, F., Rougeron, V., Becquart, P., Berry, A., Makanga, B., Rahola, N., Arnathau, C., Ngoubangoye, B., Menard, S., Willaume, E., Ayala, F. J., Fontenille, D., Ollomo, B., Durand, P., Paupy, C. & Renaud, F. 2013. Diversity, host switching and evolution of *Plasmodium vivax* infecting African great apes. *Proc Natl Acad Sci USA*, 110, 8123-8128.

Rogerson, S.J. & Carter, R. 2008. Severe *Vivax* Malaria: Newly Recognised or Rediscovered? *PLoS Med*, 5, e136.

Rottschaefer, S. M., Riehle, M. M., Coulibaly, B., Sacko, M., Niaré, O., Morlais, I., Traoré, S. F., Vernick, K. D. & Lazzaro, B. P. 2011. Exceptional diversity, maintenance of polymorphism, and recent directional selection on the APL1 malaria resistance genes of *Anopheles gambiae*. *PLoS Biol*, 9, e1000600.

Ryan, J. R., Stoute, J. A., Amon, J., Dunton, R. F., Mtalib, R., Koros, J., Owour, B., Luckhart, S., Wirtz, R. A., Barnwell, J. W. & Rosenberg R. 2006. Evidence for transmission of *Plasmodium vivax* among a duffy antigen negative population in Western Kenya. *Am J Trop Med Hyg*, 75, 575-581.

Salgueiro, P., Vicente, J. L., Ferreira, C., Teófilo, V., Galvão, A., do Rosário, V. E., Cravo, P. & Pinto, J. 2010. Tracing the origins and signatures of selection of antifolate resistance in islandpopulations of *Plasmodium falciparum*. *BMC Infect Dis*, 10, 163.

Simard, F., Licht, M., Besansky, N.J. & Lehmann, T. 2007. Polymorphism at the defensin gene in the *Anopheles gambiae* complex: Testing different selection hypotheses. *Infection Genet Evol*, 7, 285-292.

Snounou, G., Pinheiro, L., Antunes, A. M., Ferreira, C. & do Rosario, V. E. 1998. Nonimmune patients in the Democratic Republic of Sao Tome e Principe reveal a high level of transmission of *P. ovale* and *P. vivax* despite low frequency in immune patients. *Acta Trop,* 70, 197-203.

Tahar, R. & Basco, L. K. 2006. Molecular epidemiology of malaria in Cameroon. XXII. Geographic mapping and distribution of *Plasmodium falciparum* dihydrofolate reductase (dhfr) mutant alleles. *Am J Trop Med Hyg*, 75, 396-401.

Talisuna, A. O., Nalunkuma-Kazibwe, A., Langi, P., Mutabingwa, T. K., Watkins, W. W., Van Marck, E., Egwang, T. G. & D'Alessandro, U. 2004. Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated *falciparum* malaria. *Infect Genet Evol*, 4, 321-327.

Tjitra, E., Anstey, N. M., Sugiarto, P., Warikar, N., Kenangalem, E., Karyana, M., Lampah, D. A. & Price, R. N. 2008. Multidrug-resistant *Plasmodium vivax*

associated with severe and fatal malaria: A prospective study in Papua, Indonesia. *PLoS Med*, 5, e128.

Wang, X., Mu, J., Li, G., Chen, P., Guo, X., Fu, L., Chen, L., Su X. & Wellems, T. E. 2005. Decreased prevalence of the *Plasmodium falciparum* chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against *P. falciparum* malaria in Hainan, People's Republic of China. *Am J Trop Med Hyg*, 72, 410-414.

WHO 2013. World Malaria Report 2013. Geneve, Switzarland: World Health Organization.

Wondji, C., Simard, F. & Fontenille, D.2002. Evidence for genetic differentiation between the molecular forms M and S within the forest chromosomal form of *Anopheles gambiae* in an area of sympatry. *Insect Mol Biol*, 11, 11-19.